# THE DEPENDENCY OF FAITHFUL CELL DIVISION ON HYPERPHOSPHORYLATION OF ACUTE MYELOID LEUKAEMIA ASSOCIATED CYTOPLASMIC NUCLEOPHOSMIN

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## DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

_____

Chan Mei Yi Narisa
2015

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The sole candidate's name on the front page of this thesis is deceptive for, as the saying goes, it takes a village. I am but the town crier.

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# Abstract

Nucleophosmin (NPM) is a multifunctional protein that is predominantly localised in the nucleolus. However, in one-third of acute myeloid leukaemia (AML), a frame-shift mutation in the C-terminus abolishes the nucleolar localisation signal causing cytoplasmic delocalisation. This mutant, known as NPMc, does not contribute to leukaemogenesis by upregulating XIAP and cannot inhibit the intrinsic caspases 3 and 9. Instead, the centrosome-related phosphorylation sites threonine 199 and serine 4 are hyperphosphorylated on NPMc as compared to wild-type NPM which is of interest as 85% of clinical NPMc+AML have a normal karyotype upon which normal centrosome duplication is dependant. Levels of kinase and phosphatase do not differ between wtNPM and NPMc-expressing cells and phosphorylation decreases upon nuclear relocalisation indicating that cytoplasmic localisation is the primary cause of hyperphosphorylation. Furthermore, G2/M phase centrosome phosphorylation sites are not differentially phosphorylated implying that the breakdown of the nuclear envelope during mitosis abolishes the relevancy of NPM's location to its phosphorylation. Inhibition of phosphorylation leads to accumulation of cells with 4n DNA without a corresponding increase in the number of cells with 2 centrosomes, meaning that the DNA and centrosome duplication cycles are uncoupled. In conclusion, this work provides evidence that the normal karyotype in NPMc+AML is linked to the phosphorylation of NPM's centrosome-related sites paving the way for the possibility of targeting NPMc in conjunction with current chemotherapeutics to enhance their DNA-damaging and, therefore, cell death efficacy.

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**List of Abbreviations**

| | |
|---|---|
| NPM | Nucleophosmin |
| wtNPM | Wild-type NPM |
| NPMc | Cytoplasmic NPM |
| AML | Acute myeloid leukaemia |
| Sp1 | Specificity factor 1 |
| CRM1 | Chromosomal maintenance 1 |
| Cyc | Cyclin |
| Cdk | Cyclin-dependant kinase |
| Arf | Alternate reading frame protein |
| PTEN | Phosphatase and tensin homolog |
| Fbw7γ | F-box and WD repeat domain-containing 7 isoform γ |
| Plk | Polo-like kinase |
| XIAP | X-linked inhibitor of apoptosis protein |
| HEK293T | Human Embryonic Kidney 293 cells with T antigen of SV40 |
| Mdm2 | Mouse double minute 2 homolog |
| GFP | Green fluorescent protein |
| LepB | Leptomycin B |
| ROCK2 | Rho-associated kinase 2 |
| BRCA2 | Breast cancer type 2 susceptibility protein |
| PIP3 | phosphatidylinositol 3,4,5 trisphosphate |

# 1 Introduction

## *1.1 Nucleophosmin*

Identified in the late 1970's as a 37kDa nucleolic protein (Lischwe et al. 1979), nucleophosmin (NPM) has since been found to be involved in a plethora of cellular processes from ribosome biogenesis (Herrera et al. 1995) to histone chaperoning (Gadad et al. 2011). Also known as numatrin or B23 (accession no. NM_002520), this 294 amino acid protein (Figure 1.1) is the most ubiquitously expressed and well-studied member of the *nucleophosmin* gene's three splice variants which is located on human chromosome 5p35 (Falini et al. 2009). The second splice variant (NPM1.2/B23.2, accession no. NM_001037738) is expressed at minimal levels and is thought to be associated with the cytoskeleton (Wang et al. 1993). It is 259 amino acids long and lacks the 35 C-terminal amino acids of the 1$^{st}$ splice variant which contains the nucleolar localisation signal (NoLS) necessary for nucleolar localisation. As such NPM1.2 is localised to the nucleoplasm instead (Lee et al. 2007). Little is known about the third splice variant (NPM1.3/B23.2, accession no. NM_199185).

Being just outside the size limit for nuclear magnetic resonance (NMR), the structure of the whole nucleophosmin molecule is not currently known and only portions of the N- and C-terminus have entries in the protein databank (www.pdb.org).

The N-terminus of NPM (amino acids 1-122) is an eight-stranded β-barrel homo-oligomerisation domain capable of forming pentamers and decamers (Lee et al. 2007) and the majority of cellular NPM is found in multimers (Chan and Chan 1995). The C-terminal 53 amino acids of NPM, which is essential for its nucleolar localisation consists of a 3-helix bundle, joined to the rest of the protein by an unstructured 17-residue lysine-rich region (Figure 1.2).

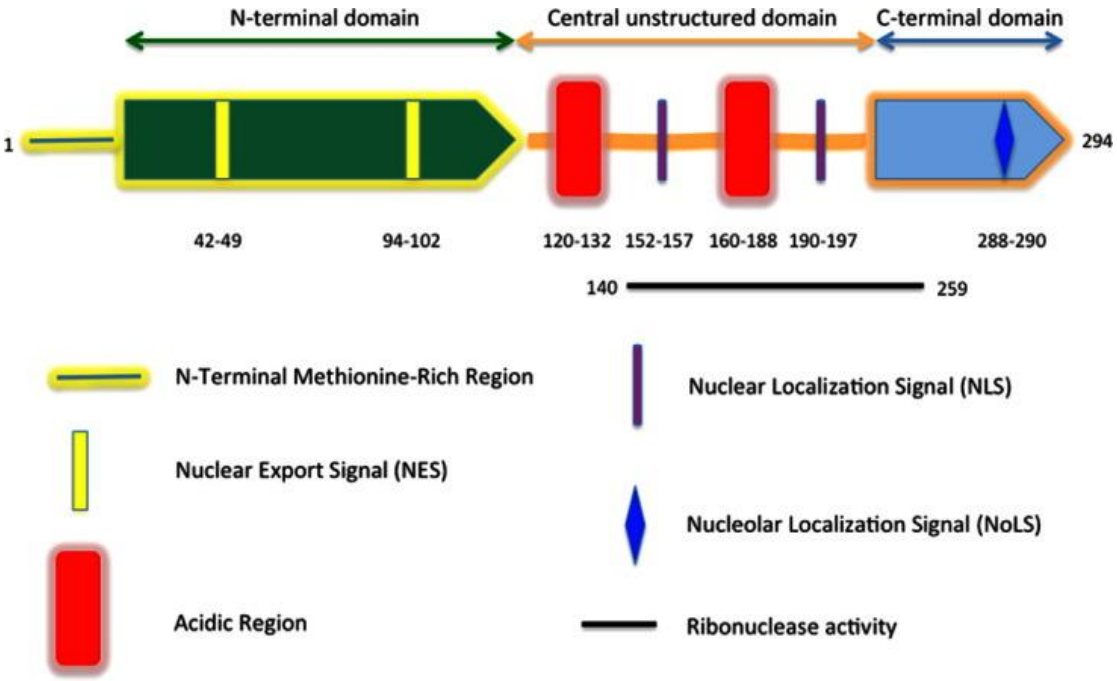


**Figure 1.1 Domain structure of NPM.**
Schematic representation of NPM and its functional domains adapted from Federici & Falini 2013.

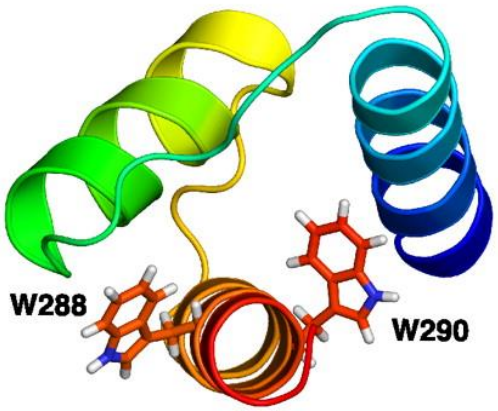


**Figure 1.2 The last 50 residues of NPM.**
PDB entry 2VXD. Highlighted are tryptophans 288 and 290 which are essential for the structural maintenance of the hydrophobic core. Adapted from Grummitt et al., 2008.

## *1.2 Cellular Functions*

### 1.2.1 Nuclear roles

#### 1.2.1.1 Nucleoli and ribosomes

As NPM was first discovered in the nucleolus, the heart of ribosome production, it is no surprise that NPM functions in ribosome synthesis. Nucleoli structural integrity is dependent on NPM S125 phosphorylation by casein kinase 2 (Szebeni et al. 2003). Mutation of S125 to non-phosphorylatable alanine leads to reticulated nucleoli and decrease rRNA transcription (Louvet et al. 2006). NPM also contains intrinsic ribonuclease activity (Savkur and Olson 1998) and knockdown of NPM inhibits pre-rRNA processing and induces cell death (Itahana et al. 2003).

During mitosis, the nucleolus breaks down and nucleolic proteins like NPM relocate to the perichromosomal layer. Some NPM remains associated with condensed chromosomes from metaphase to anaphase and decondensing chromosome in telophase. However, most NPM is indiscriminately distributed in the mitotic cell. (Hernandez-Verdun and Gautier 1994; Van Hooser et al. 2005). Furthermore, NPM is localised to the centre of mitotic spindle poles but does not participate in spindle pole formation (Zatsepina et al. 1999).

#### 1.2.1.2 DNA integrity

The nucleus contains the majority of genetic information needed for cellular function and as a predominantly nuclear protein, NPM plays a role in guarding and protecting the fidelity of DNA. During S phase, NPM binds and stabilises DNA polymerase η (polη), the loss of which is seen in the hereditary disease xeroderma pigmentosum whose increased risk of skin cancer is caused by the lack of *polη* gene which would otherwise prevent DNA double-stranded breaks and genomic instability by repairing

DNA lesions. NPM knockdown or depletion of NPM from the nucleus by the mutant cytoplasmic NPMc leads to increased proteasomal degradation of polη and decreased translesion DNA synthesis (Ziv et al. 2014).

However translesion synthesis is inherently error prone and NPM also contributes to template-dependent base excision repair (BER). NPM stimulates the BER enzyme apurinic/apyrimidinic endonuclease 1 (APE1). Knockout or cytoplasmic delocalization of NPM results in truncation and accumulation of APE1 in the cytoplasm, respectively, increasing sensitivity to genotoxic assaults (Poletto et al. 2014; Vascotto et al. 2014).

In response to DNA damage, NPM is dephosphorylated by protein phosphatase 1β which increases its interaction with the tumour suppressor retinoblastoma protein (pRB), reducing its transcription-repressing activity on E2F1. E2F1 is then upregulated and can promote the transcription of nucleotide excision repair genes RPA3, XPC and DDB2 (Lin et al. 2010).

NPM also prevents cells with damaged DNA from progressing into mitosis by binding to amino acids 61-100 of Gadd45a, maintaining it in the nucleus where it inhibits the mitotic kinase complex cdk1-cyclin B. Gadd45a is upregulated in response to DNA damage but does not have its own nuclear localization signal and is dependent on NPM for nuclear localisation. Knockdown of NPM leads to increase in cytoplasmic Gadd45a and reduced G2/M phase cell cycle arrest (Gao et al. 2005).

Finally, NPM's C-terminus stimulates DNA polymerase α activity (Umekawa et al. 2001) and promotes single-strand annealing in B-cell DNA recombination (Borggrefe 1998).

### 1.2.1.3 Transcription factors

NPM also has a role in regulating guardian of the genome, p53 (Lane 1992), directly interacting with its heterodimerization domain (amino acids 186-259). Overexpression of NPM relocalises p53 in the nucleolus instead of its usual nucleoplasmic location. This increases transcription of p53 response genes *hdm2* and *p21* and inducing cellular senescence and growth inhibition (Colombo et al. 2002). NPM also directly interacts with Hdm2, the negative p53 regulator, preventing it from interacting with p53 and thereby stabilising the latter (Kurki et al. 2004). Downstream of p53, NPM interacts with p21, reducing its ubiquitination and degradation and increasing its stability (Xiao et al. 2009).

In addition to p53, NPM can complex with other transcription factors such as NF-κB and Sp1, upregulating the expression of the antioxidant protein MnSOD (Dhar et al. 2004; Xu et al. 2007; Xu et al. 2008).

NPM also interacts with activating transcription factor 5 (ATF5), promoting its proteasome- and caspase-dependant degradation by competing with its interaction with HSP70 which stabilises ATF5 (Liu et al. 2012a).

In embryonic stem (ES) cells, NPM interacts with Oct4, Nanog and Sox2 (in ectoderm differentiation) and along with the chaperone protein Tpt1, promotes ES cell proliferation (Johansson et al. 2010; Johansson and Simonsson 2010).

### 1.2.1.4 Other roles

NPM itself is also a chaperone, binding to core histones H3, H2B and H4 by its N-terminus while the C-terminus mediates chromatin unfolding and transcription which is dependent on both histone and NPM acetylation (Swaminathan et al. 2005). On the other hand, NPM indirectly decreases chromatin accessibility by binding to high

mobility group A (HMGA), preventing it from modifying DNA and recruiting transcription factors (Arnoldo et al. 2015). Finally, NPM acts as a phosphatidylinositol 3,4,5-triphosphate receptor, preventing apoptosis by inhibiting caspase-activated DNase which causes DNA fragmentation (Ahn et al. 2005).

### 1.2.2 NPM as a shuttling/transport chaperone

In addition to its nuclear functions, NPM shuttles between the nucleolus and cytoplasm acting as a chaperone for both ribosomes and histones (Lindström 2011). Using leucines 42 and 44, NPM binds to CRM1 in order to exit the nucleus (Yu et al. 2006), bringing with it rRNA as well as the small 40S and large 60s ribosomal units (Maggi et al. 2008). NPM also regulates mRNA, limiting the length of the poly(A) adenylation tail and facilitating mRNA export. Depletion of NPM leads to hyperadenylation and accumulation of mRNA in the nucleus without greatly affecting mRNA half-life (Sagawa et al. 2011).

### 1.2.3 Cytoplasmic roles

Although predominantly localised in the nucleolus, NPM has been found to interact with the microtubule kinesin Eg5. NPM's DNA/RNA binding domain (residues 191-294) interact with Eg5's motor domain (residues 1-437), inhibiting the latter's ATPase activity and promoting microtubule polymerisation (Wang et al. 2010).

NPM also associates with the centrosome inhibiting its duplication until phosphorylated by cyclin E-cdk2 on residue T199 during mitosis (Tokuyama et al. 2001). CycE/cdk2 licenses centrosomes for duplication by phosphorylating NPM on threonine 199 (T199). This causes the dissociation of NPM away from the centrosome allowing it to duplicate. NPM remains phosphorylated throughout S/G2 phases, supposedly by being phosphorylated by cycA/cdk2 (Tokuyama et al. 2001) and only re-associates with the

centrosomes later in mitosis where it is phosphorylated by the mitotic cyclin B-cdk1 complex (Peter et al. 1990) on T234 and T237 (Cha et al. 2004). Centrosome association in mitosis is dependent on phosphorylation by the centrosome maturation kinase Nek2A and NPM remains at centrosomes until after the sister chromatids have separated in anaphase (Yao et al. 2004).

Because CycA and E both activate the same cdk (cdk2), they are functionally redundant and can compensate for each other's loss albeit with a delay in S-phase progression (Hanashiro et al. 2008). Elevated cyclin E causes premature NPM phosphorylation resulting in centrosome overduplication (Mussman et al. 2000; Nakayama et al. 2000; Saavedra et al. 2003).

NPM is also phosphorylated on serine 4 by polo-like kinase (plk) 2 in S phase and this phosphorylation is sustained through into M phase by Plk1. Mutating serine 4 to the phosphomimetic glutamate or aspartic acids results in centrosome overamplification and the opposing mutation to alanine causes elongated or multi-lobed nuclei (Zhang et al. 2004a; Krause and Hoffmann 2010).

In addition to its 37/38 kDa full-length form, NPM is known to be cleaved to 30 kDa which is further cleaved to 20 kDa to modulate cytokine production by macrophages. This is achieved primarily by caspase 7 cleavage at Asp213 producing the p30 fragment which is further cleaved by cathespin B to produce the p20 fragment (Guery et al. 2011). In natural killer (NK) cells, NPM is cleaved by granzyme M at Leu158 which facilitates NK cells' cell-killing ability, indicating a pro-survival role for NPM (Cullen et al. 2009).

### 1.2.4 Lesser known post-translational modifications

Although initially identified as a phosphoprotein (Jones et al. 1981), NPM is subject to a variety of post-translational modifications (PTMs) and as such, regularly appears in Western blots as multiple bands. NPM interacts with PARP-1 and PARP-2 and is poly-(ADP-ribosyl)ated in response to ionising radiation (Ramsamooj et al. 1995; Meder et al. 2005).

NPM is also sumoylated on lysine (K) 230 and 263. Mutation of the former site increases phosphatidylinositol-3,4,5-trisphosphate and suppresses DNA fragmentation while mutation of the latter abolishes binding to retinoblastoma protein, leads to reduced nucleolar and centrosomal localisation, and is more vulnerable to caspase-3 cleavage and apoptosis (Tago et al. 2005; Liu et al. 2007). NPM also undergoes ubiquitination, both in the canonical pathway that leads to proteasomal degradation (Itahana et al. 2003) as well as a less conventional lysine 6-linked poly-ubiquitin chain catalysed by BRCA1 E3 ligase (Sato et al. 2004). Although the exact downstream events of lysine 6-linked poly-ubiquitination are unknown, the fact that both NPM and BRCA1 are localised to centrosomes and regulated by Cdk2 suggests that a cell cycle role is likely (Hayami et al. 2005; Okuwaki 2008).

## *1.3 NPM in cancer*

### 1.3.1 Oncogenic roles

Wild-type NPM is overexpressed in a variety of carcinomas including gastric (Tanaka et al. 1992), colon (Nozawa et al. 1996), ovarian (Shields et al. 1997), breast (Skaar et al. 1998), liver (Yun et al. 2007), brain (Kuo et al. 2015) and prostate (Subong et al. 1999). NPM overexpression in proliferating cells compared to resting or differentiating cells may also drive tumourigenesis by its role in ribosome biogenesis (Maggi et al. 2008). Cancer cells exhibit enlarged nucleoli, the site of ribosome synthesis (Ruggero

and Pandolfi 2003) so the upregulation of NPM would increase the production of ribosomes which would, in turn, contribute to the elevated protein synthesis rate needed for the high rate of cellular replication in cancer cells (Grisendi et al. 2006). Overexpression of NPM can also stimulate increased RNA transcription by sequestering HEXIM1, an inhibitor of positive transcription elongation factor b (P-TEFb), which in turn transcribes RNA polymerase II (Gurumurthy et al. 2008; Lew et al. 2013).

Elevated wtNPM also inhibits apoptosis (Ye 2005), sequestering p53 in the nucleus and preventing it from localising to the mitochondria where it would otherwise activate proapoptotic proteins such as Bax an Noxa which would induce the release of cytochrome c, triggering apoptosis (Dhar and St Clair 2009). NPM itself interacts directly with Bax, preventing it from translocalising to mitochondria to facilitate mitochondrial outer membrane permeabilization (Lo et al. 2013).

In addition to inhibiting cell death, NPM is also correlated with metastatic invasiveness by enhancing the MAP kinase/ERK pathway (Liu et al. 2012b; Loubeau et al. 2014). NPM also drives DNA synthesis by binding to and stimulating DNA polymerase α activity in cooperative synergy with retinoblastoma protein (Takemura et al. 1999). Overexpression of NPM bypasses G1/S and G2/M phase checkpoints, reduces commitment to myeloid differentiation and protects against enhance survivability to DNA damage and oxidative stress (Li et al. 2006; Du et al. 2010).

NPM also enhances the activity of other oncogenes. For example, NPM interacts with the oncogenic transcription factor FOXM1 in cancer and immortal cells but not normal cells. Without the maintenance and stabilisation of FOXM1 by NPM in the nucleus, cell growth is inhibited both *in vitro* and *in vivo* (Bhat et al. 2011).

Lastly, NPM potentiates the Warburg effect by transcriptionally repressing the gluconeogenesis enzyme fructose-1, 6-bisphosphatase 1, promoting aerobic glycolysis instead of oxidative phosphorylation and thereby providing more resources for nucleic acid synthesis (Zhu et al. 2015).

### 1.3.2 NPM as a tumour suppressor

Paradoxically, NPM also exhibits properties of a haploinsufficient tumour-suppressor: hypomorphic, heteryzygous or knocked down nucleophosmin (NPM) levels cause genomic instability (Colombo et al. 2005), one of the 'enabling characteristics' of tumourigenesis (Hanahan and Weinberg 2011). NPM+/- mice develop myeloid and lymphoid malignancies and deletion of chromosome 5q arm, where the NPM gene is situated, is frequently found deleted in lung cancers (Mendes-da-Silva et al. 2000; Sportoletti et al. 2008). It seems therefore that NPM exhibits the "goldilocks" trait: cellular homeostasis requires an intermediate amount of NPM and too much or too little leads to oncogenesis.

### 1.3.3 Haematological cancers

NPM is also implicated in haematological malignancies, where consistent and recurrent clonal chromosomal aberrations are more common than in solid tumours (Kaye 2009). Approximately 80% of all cancers with clonal chromosomal abnormalities are blood cancers. The most famous, or perhaps, infamous of these is the Philadelphia chromosome of chronic myeloid leukaemia (Figure 1.3). In this classic textbook example, the translocation of the q arms of chromosomes 9 and 22 cause a gene fusion product with increased tyrosine kinase activity (Mitelman et al. 2007). This leads to activation of multiple signal transduction pathways that contribute to many of the hallmarks of cancer: reduced apoptosis, decreased dependence on growth factors,

impaired interaction with stroma and extracellular matrix and increased proliferation (Deininger et al. 2005).

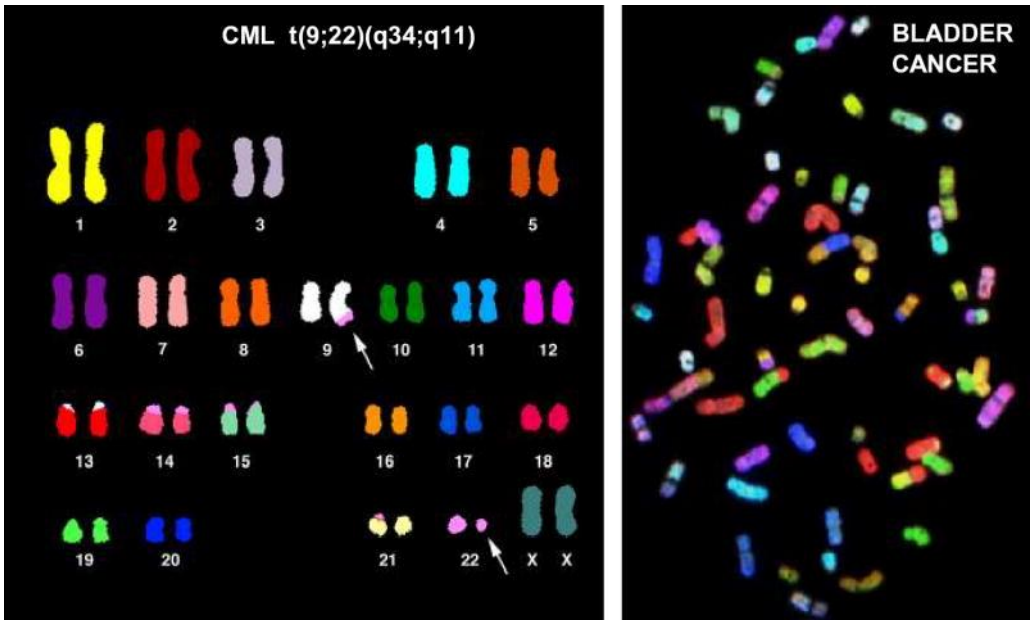


**Figure 1.3 Chromosome aberrations in cancers**
Cytogenetic analysis can often reveal recurrent abnormalities that provide a 'smoking gun' that is sufficient for diagnosis (pathognomonic). Left panel, white arrows, translocation of chromosomes 9 and 22 found in the vast majority of chronic myeloid leukaemia (CML); right panel, example of the genetic anarchy of a solid tumour (bladder cancer). Adpated from Aplan, 2006.

In about 50% of anaplastic large cell lymphoma, the N-terminus of NPM (which contains an oligomerisation domain) is fused with the C-terminus of anaplastic lymphoma kinase (which contains the kinase domain) due to a t(2;5)(p23;q35) chromosome translocation. This causes oligomerisation and activation of the kinase and downstream signalling leading to lymphomagenesis. Much rarer are t(5;17) and t(3;5)(q25;q35) chromosomal translocations which are found in acute promyelocytic leukaemia and acute myeloid leukaemia, respectively. The former results in an NPM-retinoic acid receptor-α fusion that inhibits differentiation while the latter causes NPM-myeloid leukaemia factor 1 fusion whose role in leukaemogenesis remains to be determined (Falini et al. 2007).


**Figure 1.4 The N-terminal portion of NPM**
The oligomerisation domain (residues 1-110) is found with gene fusions in various blood tumours. Adapted from Lim & Wang, 2006.

## *1.4 Acute Myeloid Leukaemia*

Acute myeloid leukaemia or AML is a heterogeneous haematological malignancy which is diagnosed as more than 20% immature/undifferentiated myeloblasts in the blood or bone marrow according to the World Health Organisation (Hasserjian 2013). Symptoms include haemorrhages, weakness, anaemia and susceptibility to infections (Moore 1964) and treatment involves leukaepheresis, bone marrow transplantation and chemotherapy (Grimwade et al. 2001). AMLs can be classed according to the morphology of the myeloblasts as is done in the French-American-British classification system (de Jonge et al. 2011). However, the WHO has proposed a revised system that is more dependent on karyotype and gene mutations which are more useful and relevant for prescribing treatment and establishing prognosis (Vardiman et al. 2009).

## *1.5 NPMc*

Specific to around one-third of acute myeloid leukaemia (AML) cases is a cytoplasmic form of nucleophosmin with a frameshift mutation in the 12$^{th}$ and final exon (Falini et al. 2005).

| Type of Mutation | GenBank Accession No. | Sequence | Predicted Protein |
|---|---|---|---|
| None (wild type) | NM_002520 | GATCTCTG....GCAGT....GGAGGAAGTCTCTTTAAGAAAATAG | -DLWQWRKSL |
| Mutation A | AY740634 | GATCTCTGTCTGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG | -DLCLAVEEVSLRK |
| Mutation B | AY740635 | GATCTCTGCATGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG | -DLCMAVEEVSLRK |
| Mutation C | AY740636 | GATCTCTGCGTGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG | -DLCVAVEEVSLRK |
| Mutation D | AY740637 | GATCTCTGCCTGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG | -DLCLAVEEVSLRK |
| Mutation E | AY740638 | GATCTCTG....GCAGTCTCTTGCCCAAGTCTCTTTAAGAAAATAG | -DLWQSLAQVSLRK |
| Mutation F | AY740639 | GATCTCTG....GCAGTCCCTGGAGAAAGTCTCTTTAAGAAAATAG | -DLWQSLEKVSLRK |

**Figure 1.5 NPMc's frame-shift mutation.**
Alignment of the four-base insertions (red letters), their effects on amino acid sequence and percentage out of 52 NPMc cases. Additional amino acids in green highlight, tryptophan resideus in yellow and substituted residues in grey. Reproduced with permission from Falini et al. 2005, Copyright Massachusetts Medical Society.

Although the exact sequence and location of the 4-base insertion may vary between patients, the end result is the same (Figure 1.5): The abolition of tryptophans 290 and/or 288 which causes a disruption of the hydrophobic core needed to maintain of the tertiary structure of the C terminus and NoLS (Grummitt et al. 2008) as well as the acquisition of a nuclear export signal (NES) at the C-terminus, in addition to one already present at residues 94-102 (Falini et al. 2006). As such, NPMc is 4 amino acids longer (298 residues) than wtNPM (294 residues). Evidence shows that this unstructured C-terminal domain has a strong tendency to form amyloid-like aggregates, although how this could translate to the (dys)function of NPMc in the cell remains to be elucidated (Di Natale et al. 2015).

The mutation is stable as can be seen in the NPMc-expressing cell line OCI-AML3 and in by comparing de-novo AML samples with relapsed cells from the same patient (Meloni et al. 2009). A small proportion of chronic myelomonocytic leukaemia also display the NPMc mutation and these patients are more likely to progress to AML and have lower survival rate (Peng et al. 2015).

Despite the heterozygous nature of the mutation, the oligomerisation domain at the protein's N-terminus, which remains functional, binds to wild-type NPM (wtNPM) and exports it out of the nucleus in a dominant negative manner. Overexpression of wtNPM increases the NoLS load on an oligomer and can rescue NPMc back to the nucleolus (Bolli et al. 2009).

Approximately 85% of NPMc-positive AML cases are cytogenetically normal (Estey 2010) and it is more common in female adult patients (Falini et al. 2007). It is associated with increased stem cell maintenance *HOX* gene expression (Alcalay et al. 2005) and is prognostically favourable in the absence of *FLT3*-Intenal Tandem Duplication (ITD) mutation. In general, the *FLT3*-ITD mutation is prognostically unfavourable but in older patients (>60 years of age), the presence or absence of NPMc has more influence on prognostic survival (Becker et al. 2010; Röllig et al. 2010). Furthermore, NPMc+ patients are twice as likely to have the *FLT3*-ITD mutation that those with wtNPM (Rau and Brown 2009).

With multiple protein-interaction partners, the cytoplasmic delocalisation of NPM is bound to have pleiotropic effects and there are various ways NPMc contributes to leukaemogenesis. NPMc+AML displays unique and distinct miRNA and mRNA profiles (Garzon et al. 2008) and has a reduced ability to protect the tumour suppressor ARF from degradation (Figure 1.6), leading to a dampened p53 response (Colombo et al. 2006). ARF binds to the p53 ubiquitinator, Hdm2, and promotes its degradation thereby stabilising p53 (Zhang et al. 1998).

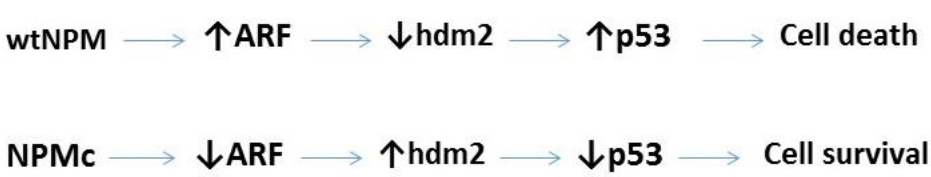


**Figure 1.6 NPMc supresses p53 and enhances cell survival**

### 1.5.1 Location, location, location

The old property agent's mantra seems to be a common theme for NPMc's oncogenic role: the C-terminal frame-shift mutation appears to affect the localisation of cellular NPM and little else; its binding ability and dynamics with other various protein partners remain largely unchanged and it is the mislocalisation of these protein partners that cause carcinogenesis.

For example, HAUSP (herpes virus-associated ubiquitin specific protease) is a deubiquitinator of PTEN whose tumour suppressor function is dependent on its nuclear localisation which is facilitated by its monoubiquitination. Both wtNPM and NPMc can bind to HAUSP but in the former case, wtNPM is maintaining HAUSP in the nucleus where it sequesters HAUSP and prevents it from deubiquitinating PTEN. PTEN then remains active in its monoubiquitinated state in the nucleus. In NPMc+ cells, there is less NPM in the nucleus to inhibit HAUSP so PTEN becomes deubiquitinated and leaves the nucleus into the cytoplasm where it is polyubiquitinated. Any HAUSP brought into the cytoplasm by NPMc is also sequestered and inactivated leading to accumulation of polyubiquitinated PTEN and its eventual degradation (Noguera et al. 2013).

Similarly, both wtNPM and NPMc can bind to the ubiquitin ligase Fbw7γ. In the nucleus, Fbw7γ ubiquitinates the myc oncogene for degradation but in NPMc+ cells, Fbw7γ is exported into the cytoplasm where it is degraded, increasing myc's stability as well as the transcription of myc target genes (Di Fiore 2008; Bonetti et al. 2008).

Miz1, a transcription factor for cell cycle inhibitors p15 and p21, requires wtNPM as a co-activator. NPMc retains its binding ability to Miz1 but exports it to the cytoplasm where is it degraded thereby reducing cell cycle arrest and promoting cell growth (Wanzel et al. 2008).

In cell death, NPM can inhibit caspases 6 and 8 but wtNPM's nucleolar localisation prevents this from having a significant effect on the cell. NPMc, however, is localised to the cytoplasm where the caspases are. NPM's interaction domains with the caspases are towards the N-terminus of the protein and do not seem to be affected by NPMc's mutation at the C-terminus. It is this retention of caspase inhibition ability that inhibits the extrinsic cell death response and promotes cell survival (Leong et al. 2010). NPMc's inhibition of caspases 6 and 8 also suppresses myeloid differentiation. In both mouse and zebrafish models, overexpression of NPMc leads to accumulation of hematopoietic progenitor cells (Bolli et al. 2010; Cheng et al. 2010) and downregulation of NPM leads to increased caspase 6 and 8 activity, differentiation and apoptosis (Yi et al. 2015).

This concept of opposing effects in the nucleus versus cytoplasm is not new. p53 is also known to have a 'Janus' personality depending on its subcellular location; inhibiting autophagy and apoptosis by caspase 9 in the cytoplasm (Tasdemir et al. 2008; Callen 2013; Chee et al. 2013) but promoting transcriptional activation of autophagy and apoptotic genes in the nucleus (Brady and Attardi 2010).

## *1.6 Mitosis*

Mitosis is known as the process by which cells divide their duplicated genetic material between two daughter cells. But in addition to DNA, other subcellular organelles are subject to this division as well, either stochastically during cytokinesis or in coordination with cytoskeletal structures. The former strategy is applicable to high-copy number organelles such as mitochondria while low-copy or single-copy organelles like the centrosome or Golgi apparatus tend to opt for the latter strategy. Daughter cells' organelles therefore are not usually made de novo but are multiplied from pre-existing

templates inherited from the mother cell (Nunnari and Walter 1996). Let's look at how some of this is achieved.

### 1.6.1 Endoplasmic reticulum and Golgi apparatus

While the endoplasmic reticulum (ER) was initially thought to fragment and be distributed stochastically (Warren and Wickner 1996), recent studies have shown that (Lu et al. 2009), at least in some cells, ER is partitioned by association with cortical actin at the plasma membrane and also with microtubules while still maintaining its cisternal structure (Figure 1.7).

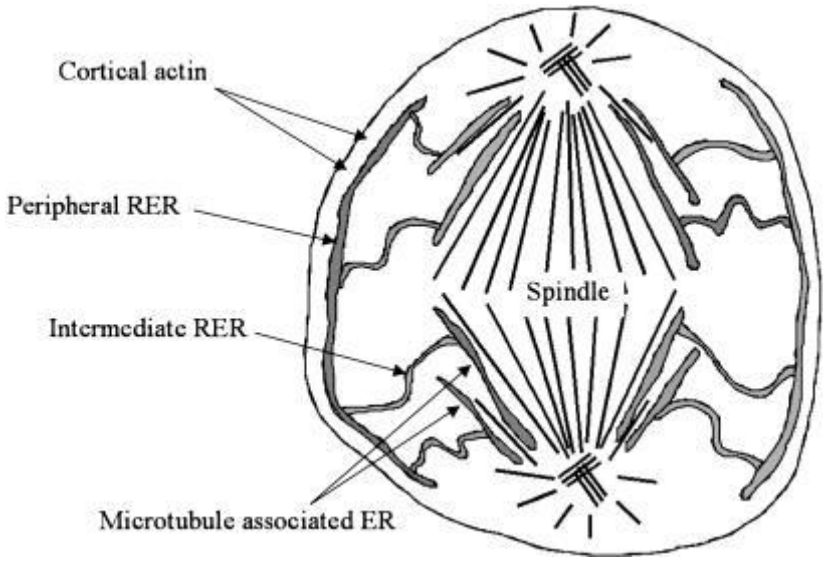


**Figure 1.7 Distribution of endoplasmic reticulum during mitosis.**
ER, endoplasmic reticulum; RER, rough ER. Adapted from McCullough & Lucocq 2005.

There is less consensus on the distribution of the Golgi. While some observe the fragmentation of the Golgi and subsequent stochastic partitioning (Cabrera-Poch et al. 1998), others observe the absorption of the Golgi into the ER which reconstitutes the former upon completion of mitosis (Zaal et al. 1999). This discrepancy may result from the use of different Golgi makers as some Golgi proteins may coalesce with the ER while others remain associated with Golgi fragments (Rabouille and Jokitalo 2003).

### 1.6.2 Endosomes and lysosomes

Endosomes are the mechanism by which cells uptake larger macromolecules from their environment and usually lead to metabolic degradation in fusion with lysosomes. The migration of endosomes appears to be dependent on their maturity. Early endosomes are positioned around centrosomes, away from the metaphase plate, during metaphase. As anaphase proceeds, they migrate over to the minus ends of shortening microtubules and adopt a juxtaposed position to the centrosome as the nuclear membrane reassembles. On the other hand, late endosomes and lysosomes are less ordered and are more passively distributed (Dunster et al. 2002).

### 1.6.3 Mitochondria

Despite being a high-copy number organelle, there is evidence to show that mitochondria are actively localised to the cleavage furrow during mitosis (Lawrence and Mandato 2013a). Inhibition of microtubules with nocodazole or taxol abolished the accumulation of mitochondria at the cell's equator and the authors suggest that this localisation may be needed to meet the energy requirement for cytokinesis (Lawrence and Mandato 2013b).

### 1.6.4 Nucleoli

With the dissolution of the nucleus during mitosis, the structure of the nucleoli is also disassembled. The nucleolus is organised around ribosome biogenesis with fibrillar centres in which the ribosomal DNA are housed, the dense fibrillary component where rRNAs are processed and further processing and ribosome assembly in the outer most granular component (Hernandez-Verdun et al. 2010). RNA transcription begins to decrease at the start of prophase and cease by anaphase along with the dissociation of nucleolar proteins including NPM (Gébrane-Younès et al. 1997; Leung et al. 2004). Furthermore, the phosphorylation of NPM by cdk1 on at least four threonine residues

in its ribonuclease domain (T199, T219, T234, and T237) prevent its rRNA binding ability and nucleolar localisation (Okuwaki et al. 2002; Negi and Olson 2006). Nucleolar components then surround condensed chromatin, forming a perichromosoal layer around chromosomes. How this layer is maintained is poorly understood but hitchhiking onto the precisely coordinated separation of DNA ensures that nucleolar components are likewise equally distributed (Hernandez-Verdun 2011).

As can be seen, microtubules not only govern the partitioning of DNA during mitosis but also that of other subcellular organelles. However, one hardly hears of diseases which are caused by inaccurate distribution of other organelles. DNA, however, is the source of genetic information from which other cellular machinery is constructed so defects in DNA replication or distribution become the source of mutations that persist in diseases such as cancer.

## *1.7 Centrosomes*

Cancer has been described as a microevolutionary process, not least because it defies the cooperation and collaboration that is characteristic of multicellular organisms (Alberts et al. 2002). It has been suggested that, in order to overcome the defences setup to stop such selfish behaviour, genome instability develops early in cancer development as a means to get rid of tumour suppressors and activate oncogenes (Boland and Ricciardiello 1999). One source of genome instability is centrosome dysfunction. The small size (1-2μM, Fukasawa 2005) of this organelle betrays the complexity of its duplication and its importance in the faithful segregation of chromosomes in mitosis.

### 1.7.1 Structure and organisation

The centrosome consists of two fibre-linked centrioles surrounded by a number of proteins collective called pericentriolar material (Figure 1.8). One centriole containing

appendages which are needed for microtubule nucleation is known as the mother centriole and the other, the daughter centriole. It is only just before mitosis that the daughter centriole acquires the appendages for spindle pole formation (Bornens 2002).

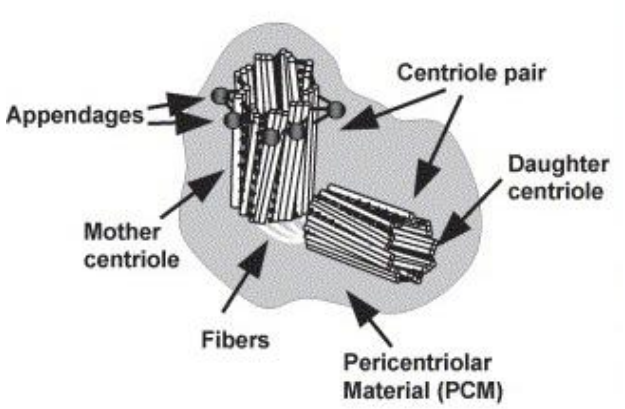


**Figure 1.8 Structure of a centrosome**
Diagram of centrioles surrounded by PCM. Adapted from Fukasawa 2005.

### 1.7.2 Duplication

Like DNA replication, centrosome duplication is semi-conservative, triggered by cdk2 and has strict controls to ensure that it only occurs once per cell cycle (Blow and Dutta 2005; Tsou and Stearns 2006b; Tsou and Stearns 2006a).

#### 1.7.2.1 Disengagement and licensing

Duplication actually begins at the end of the previous M phase with centriole disengagement in which the orientation between the mother and daughter centrioles is loosened. Although the mechanisms of various factors have yet to be resolved, it is generally agreed upon that separase-mediated cleavage of cohesin is necessary but not sufficient (Nakamura et al. 2009; Schöckel et al. 2011) for disengagement. This makes sense as cohesin also holds sister chromatids together and their separation occurs much earlier in M phase. Pericentrin B (PCNTB)/kendrin has been proposed to be one of the additional factors needed to be cleaved by separase at R2231. The resulting short C-

terminal fragment is released and degraded while there is a delay in the dissociation of the N-terminal fragment, supposedly via oligomerisation with as yet uncleaved PCNTB (Matsuo et al. 2012; Lee and Rhee 2012).

However, the knockdown of separase leads to only a delay in centriole disengagement as polo-like kinase 1 (Plk1) eventually overcomes this. The total failure of centriole disengagement requires knockdown of both separase and plk1 (Tsou et al. 2009). In addition, Plk1 further participates in the next step of centrosome duplication (licensing) by phosphorylating NPM on serine 4. It is centrosome licensing that NPM was first implicated in (Section 1.2.3) and it is centrosome licensing that ensures duplication is only initiated once per cell cycle (Nigg 2007).

Nevertheless, it would be ignorant to presume that NPM is the sole licenser. Mps1 is also phosphorylated by cdk2, stabilising it at centrosomes and promoting duplication. Mps1 is so named because yeast cells lacking it entered mitosis with monopolar spindles (Winey 1991; Pike and Fisk 2011). Mutation of the cdk2 phosphorylation site T468 to alanine results in proteasome-dependant removal of Mps1 from centrosomes (Kasbek et al. 2007). This explains why overexpression of Mps1 in itself is insufficient to trigger centrosome reduplication because Mps1 can still be moved from the centrosome into the cytoplasm (Pihan 2013).

Plk4 also licenses centrosomes. Like plk1, plk4 activity peaks during mitosis. During interphase, plk4 levels are kept low during interphase via autophosphorylation which triggers ubiquitination and subsequent proteasome degradation (Holland et al. 2010; Guderian et al. 2010). Mutation of phosphorylation sites leads to plk4 accumulation and centrosome overduplication, triggering p53-dependant cell-cycle arrest (Holland et al. 2012).

### 1.7.2.2 Nucleation and elongation

After licensing, plk4 then initiates procentriole nucleation. Overexpression of plk4 induces multiple procentrioles instead of only just one which does not affect the mitosis immediately after but carries the centrosome amplification phenotype into the next cell cycle. Spindle assembly abnormal 6 (Sas-6) acts upstream of plk4 as Sas-6 knockdown abolishes procentriole assembly even in the presence of plk4. Plk4 subsequently recruits other factors such as γ-tubulin and CPAP (putative homolog of *C. elegans* Sas-4) (Kleylein-Sohn et al. 2007). Sas-6 is crucial to forming what is known as the cartwheel assembly by self-assembling into a nine-spoked wheel (van Breugel et al. 2011), each spoke consisting of an Sas-6 homodimer 40˚ apart (Figure 1.9). In mitosis, anaphase-promoting complex in association with the adaptor protein Cdh1 ubiquitinates Sas-6's C-terminus, lowering cellular levels of Sas-6 to prevent extraneous procentriole nucleation (Strnad et al. 2007).

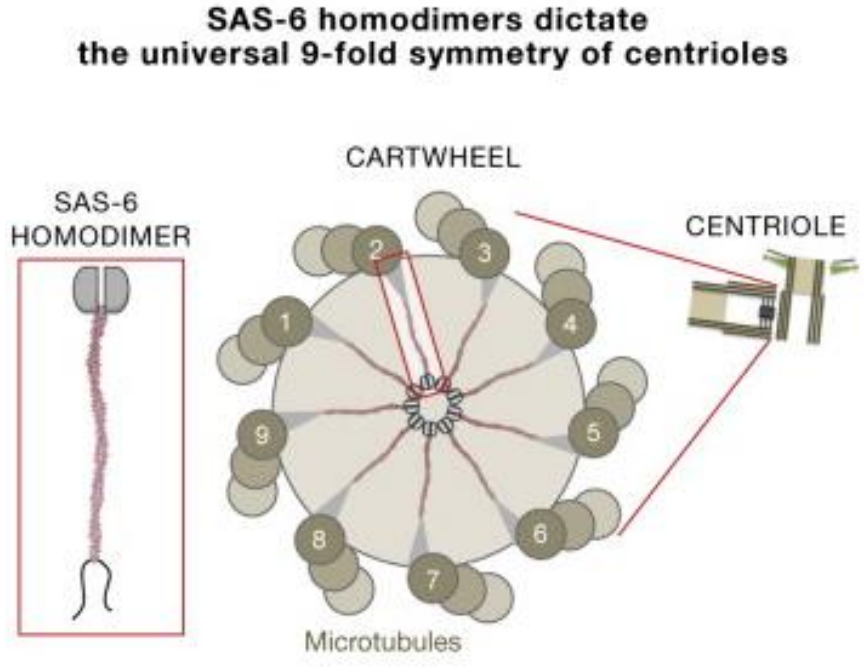


**Figure 1.9 SAS-6 forms the basis of centriole's nine-fold symmetry.**
Sas-6's N-terminus forms the central ring of the cartwheel assembly whose lumen is 23nm wide. The central coiled-coil domain forms spokes that radiate outwards to the C-terminus

where other centriole factors bind and is also the location of ubiquitination leading to degradation. Adapted from Kitagawa et al. 2011.

Now that the new centriole has been nucleated, addition factors such as γ-tubulin and its interaction partner NEDD1 (neural precursor cell expressed, developmentally down-regulated 1) are recruited to elongate it during S to G2 phase (Haren et al. 2006). CPAP (centrosomal P4.1-associated protein) also participates in elongation (Tang et al. 2009). Overexpression of CPAP leads to long centrioles which recruit extra pericentriolar materials which form extra procentrioles (Kohlmaier et al. 2009). Elongation is capped by centriolar coiled-coil protein of 110 kDa also known as centrosomal protein of 110 kDa (CP110). Depletion of CP110 leads to elongated centrioles (Chen et al. 2002) so during late S to G2/M phase, CP110 is stabilised by interaction with the deubiquitinating enzyme USP33 which prevents CP110 degradation (Li et al. 2013).

### 1.7.2.3 Maturation

With the completion of centriole growth, the next step is to accumulate pericentriolar material (PCM) and dissolve the link between the two centrosomes so that they may migrate to opposing ends of the cells to separate chromatids. PCM accumulation is largely controlled by plk1 which phosphorylates NEDD1 at serine 418 to activate its γ-tubulin recruiting capability. Recruitment of other PCM factors such as Cep192 (centrosomal protein of 192 kDa), pericentrin and Cep215 are also dependant on plk1 activity (Haren et al. 2009).

Unlike centriole disengagement, dissolution of the intra-centrosomal link is not facilitated by cleavage of the fibres but by evicting linker components. This is controlled by Nek2 (NIMA-related kinase 2) phosphorylation of fibre components.

Nek2 overexpression leads to premature centrosome separation (Fry et al. 1998) and siRNA knockdown prevents it, causing mitotic delay (Fletcher et al. 2005).

Most importantly, plk1 recruits the motor protein Eg5 to centrosomes to facilitate bipolar movement of the duplicated centrosomes. Without functional Eg5, mitosis is halted in prometaphase due to monopolar spindles (Whitehead and Rattner 1998; Kapoor et al. 2000).

The above is a greatly simplified reduction of the centrosome duplication process, without which it would not be possible for the cell to divide. Owing to its inhibitory role in centrosome duplication, one would think that the accumulation of NPM in the cytoplasm would prevent cellular division. Yet NPMc+ AML is able to overcome this and become leukaemogenic. Clearly, the advantages of cytoplasmic delocalisation outweigh any compensatory mechanism needed to permit centrosome duplication and this thesis proposes that phosphorylation of NPM is that very compensatory mechanism that allows the cell cycle to proceed. So it is perhaps ironic that the path that lead to this discovery started with the investigation of what can be considered the antithesis to cell division: cell death.

## *1.8 Cell death*

In addition to increasing the rate of cell proliferation, the cancer progenitor must also evolve ways to avoid following commands to die, whether they originate intracellularly or externally from other cells. Apoptosis is arguably the most well-defined method of cell death and is contingent on cysteine aspartyl-specific proteases or caspases (Testa and Riccioni 2007). As their name implies, caspases are cleaved at specific aspartate residues turning the inactive zymogen into an activated form. Caspases are classed into initiator (caspases 8, 2, 9 and 10) and effector (caspases 3, 6 and 7) caspases. The former

are unique to whether the death signal originated from transmembrane receptors or internally by compromising mitochondrial integrity. Both sources of initiator caspases converge on effector caspases.

### 1.8.1 Extrinsic apoptosis

Extrinsic apoptosis is triggered via activation of transmembrane receptors by external ligands such as FasL, tumor necrosis factor (TNF) and TNF-related apoptosis-inducing ligand (TRAIL) and these can be harnessed in anti-cancer therapy. Of these three listed above, TRAIL is the most likely candidate as it does not induce undesirable side effects (Ogasawara et al. 1993; Bazzoni and Beutler 1996). Unfortunately, most cases of AML are TRAIL-resistant (Wuchter et al. 2001; Testa and Riccioni 2007) mediated by TRAIL receptor downmodulation or mutation of the downstream caspase 8 (Suh et al. 2003). Co-treatment with drugs that enhancing TRAIL response have shown some promise but are yet untested in the clinic (Hasegawa et al. 2006; Szegezdi et al. 2006). Downstream of extrinsic transmembrane receptor is the adaptor molecule Fas associated protein with death domain (FADD) which possess another barrier to cell death. Levels of FADD are positively correlated with prognosis and (Tourneur et al. 2004) so even if the upstream receptor is expressed/functioning, the signal may not be transmitted further is there is not enough FADD to respond (Lewis et al. 2000). Alternatively, FADD may be counteracted by FADD-like inhibitory proteins (FLIP) which bind to FADD and competitively inhibit binding to pro-caspase 8 which would otherwise activate it (Tschopp et al. 1998). Caspase 8 may itself be altered to caspase 8L which lacks the protease domain but still maintains binding ability to FADD, acting in a similar fashion to FLIP (Himeji 2002; Mohr et al. 2005).

Interestingly, caspase 8 is also involved in another form of cell death called necroptosis. The name derives from the fact that it is morphologically like necrosis but is controlled by signalling like apoptosis. In this pathway, inactive caspase 8 forms a complex with FADD, recruiting receptor-interacting proteins (RIP) 1 and 3 which potentiate downstream signalling, causing fission of mitochondria and increased reactive oxygen species. Conversely, active caspase 8 cleaves RIP1/3, favouring the apoptotic response in the absence of caspase inhibition (Vandenabeele et al. 2010). Active caspase 8 also mutes necroptosis by cleaving cylindromatosis (CYLD) which would normally deubiquitinated RIP1 so that it is available to form the necroptosome (O'Donnell et al. 2011). Because NPMc inhibits caspase 8, one would expect necroptosis to be potentiated. However, recent work in the lab that this alternative death pathway is also inhibited by NPMc. RIP1 cleavage is elevated in NPMc expressing cells although further work is needed to elucidate the mechanism (Bte Ahmad 2014).

### 1.8.2 Intrinsic (mitochondrial) apoptosis

The energy factory of the cell is, perhaps, ironically also the trove of pro-apoptotic effectors such as cytochrome c, Smac/DIABLO, Omi/HtrA2 and AIF which are released upon irreparable cellular damage or unfavourable environmental growth conditions (Ishizaki et al. 1995; Fulda and Debatin 2006). This occurs by proteins which open pores in the mitochondrial membrane from the Bcl2 family.

In AML, upregulation of anti-apoptotic Bcl-2 members such as Bcl-2 (Campos et al. 1993; Klampfer et al. 1996; Traver et al. 1998) and Bcl-$X_L$ (Konopleva et al. 2002; Minami et al. 2003; Bagrintseva et al. 2005) as well as inactivation of pro-apoptotic proteins such as Bad and Bax (Martelli et al. 2006) is a common strategy to avoid cell death. Moreover, a high Bcl-2 to Bax ratio is associated with expression of the stem

cell marker CD34 (van Stijn et al. 2003). Bcl-2 may be upregulated by the transcription factor CCAAT/enhancer binding protein α (C/EBPα) which is mutated in 10% of AML. These mutants, lack either the N-terminal transactivation domain or the C-terminal DNA-binding domain, cooperate with nuclear factor-κB (NF-κB) p50 to transactivate Bcl-2 (Paz-Priel et al. 2005). Therefore, it is no surprise that Bcl-2 is a potential druggable target in the clinic (de Lima et al. 2004; Dai et al. 2005; Marcucci et al. 2005). Bcl-2 prevent mitochondria membrane permeabilisation and a major consequence of this is the release of cytochrome c which goes on to form the apoptosome complex with apoptotic protease activating factor 1 (Apaf1) and caspase 9. Leukaemic cells have been shown to have low Apaf1 levels due to DNA methylation and treatment with the methylation inhibitor 5-aza-2'-deoxycytidine increased Apaf1 levels (Furukawa et al. 2005).

The downstream caspase of the apoptosome, caspase 3, is correlated with poor prognosis (Estrov et al. 1998) and found to be elevated in relapsed AML but not *de novo* cases indicating that it is a secondary event and not involved in the primary leukaemogenesis (Gronda et al. 2005).

The major caspase inhibitor of the intrinsic pathway is X-linked inhibitor of apoptosis protein (XIAP) which is found to be correlated with poor survival (Tamm et al. 2004b; Tamm et al. 2004a) more so than survivin, another caspase inhibitor (Adida et al. 2000). Small molecule inhibitors of XIAP are able to decrease XIAP levels and sensitise AML cells to TRAIL-induced apoptosis (Carter et al. 2005; Carter et al. 2006; Carter et al. 2008).

The first chapter of results (chapter 3), therefore, entertains the possibility that NPMc may play a role in XIAP regulation.

## *1.9 Objectives*

In the course of investigating XIAP regulation, it was noted that the centrosome-related phosphorylation site threonine 199 was more phosphorylated on NPMc than wtNPM. This warrants further investigation as T199 phosphorylation is a pre-requisite to centrosome duplication and unphosphorylated NPM inhibits centrosome duplication. Correct timing and amount of phosphorylation is critical for maintaining normal karyotype and incorrect centrosome duplication can lead to genomic instabilities such as aneuploidies commonly found in cancer. Coincidentally, 85% of NPMc cases have a normal karyotype. Therefore we hypothesized that the hyperphosphorylation of NPMc was somehow related to the fact that a greater proportion of subcellular NPM was now localized in the cytoplasm and hence has greater accessibility to the centrosome.

Hence, the remaining majority of this thesis aims to look into how this elevated phosphorylation is achieved and how it affects centrosome duplication and cell division.

# 2 Methods and Materials

## *2.1 Cell Culture*

HEK293T cell lines (a kind gift from Dr Low Boon Chuan, NUS) were cultured in RPMI 1640 (Sigma, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA) and 1% penicillin/streptomycin (PS, from PAA Laboratories GmbH, Austria). OCI-AML2 and 3 cell lines were cultured in alpha-Modified Eagle's Medium (αMEM, Sigma, USA) supplemented with 20% FBS and 1% PS.

Cells were maintained in a 37°C incubator with a humidified atmosphere of 5% $CO_2$ and passaged at 90% confluence.

## *2.2 Drug treatment*

Cells were treated for 16 hours with 10μM of GW8510 (Sigma, USA) in dimethyl sulfoxide (DMSO), 4 hours with 10nM of leptomycin B in 70% methanol solution as provided by Sigma, USA. Cycloheximide was dissolved in DMSO and used at 100μg/ml and 10μg/ml for HEK239T and OCI-AML2/3 cells, respectively.

## *2.3 S100 cell lysis*

For GST pulldown, HEK293T cells that had been transfected (Section 2.7) with were pelleted at 2000 revolutions per minute (rpm)and washed once with 1x PBS and resuspended in 100μl of S100 buffer [20mM Hepes-KOH, pH 7.5, 10mM KCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA, 3mM dithiothreitol, 1% protease inhibitor cocktail (Roche Molecular Biochemicals, USA)]. The cell suspensions were then sonicated on ice in 1sec pulse, 1sec rest cycles for a total of 30 cycles. Samples were then centrifuged at 20,000g for 20min. The supernatant was collected and protein concentration was estimated by measuring absorbance at 280nm using the *Thermo Scientific* NanoDrop *2000*™ Spectrophotometer.

## 2.4 GST pulldown

NPM proteins that had previously been cloned into pGEX4T-3 were transformed into *Escherichia coli* (*E. coli*) BL21 and cultured overnight in 8ml of Luria-Bertani (LB) broth with 100μg/ml ampicillin (Sigma, USA). The culture was upscaled to 80ml and 0.1M isopropyl β-D-1-thiogalactopyranoside (IPTG) was added 6 hours later to activate expression of GST proteins. After overnight expression, bacteria was pelleted and resuspended in 10ml of binding buffer [1% Triton-X in PBS, 1% protease inhibitor cocktail (Roche Molecular Biochemicals, USA)]. Cells were lysed by sonication on ice at 40% amplitude for a total of 4mins in 9secs pulses with 9secs of rest in between pulses and then centrifuged at maximum speed (8500rpm) for 15mins. The supernatant was incubated with 25ul of glutathione sepharose 4B beads (GE, USA), that had been pre-washed once with PBS and once with binding buffer, for 1hour at room temperature with end-over-end rotation. Beads were then centrifuged at 500g for 5mins and washed twice with binding buffer and once in S100 buffer before overnight incubation at 4°C with 5mg of HEK293T S100 lysate as described above. The GST beads were pelleted the next morning and the excess supernatant lysate was removed. Beads were washed 5 times with binding buffer before being boiled in SDS-PAGE loading buffer (62.5mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 2% w/v bromophenol blue) at 95˚C. A total of 100μg of S100 lysate was loaded as input.

## 2.5 Total Cell lysis

Cells from 6-well plates were centrifuged at 2000rpm for 5 min and washed once with PBS. The pelleted cells were resuspended in 100μl of hot SDS lysis buffer [100mM Tris-HCl pH8.0, 2% sodium dodecyl sulphate (SDS), 50mM dithiothreitol (DTT), 20% glycerol] and incubated at 85°C for 10mins. Samples were then sonicated at 40% amplitude for 15 s in 1 s pulses with 1 s of pause after each pulse followed by another

10 min incubation at 85°C. Protein concentration was estimated by measuring absorbance at 280nm using the *Thermo Scientific* NanoDrop *2000*™ Spectrophotometer.

## *2.6 Electrophoresis and Western Blot*

Proteins were separated under denaturing conditions in 12% polyacrylamide gels and electroblotted onto nitrocellulose membranes using Trans-Blot SD semi-dry transfer (Bio-Rad, USA) at 15V for 35 min. Membranes were blocked with 3% w/v skimmed milk in Tris buffered saline (TBS) with 0.1% v/v Tween-20 (TBST) for 15mins followed by overnight incubation with primary antibodies diluted in 5% w/v bovine serum albumin (BSA) in TBS. Mouse anti-cNPM (Developmental Biology Lab, NUS) and mouse anti-NPM (Invitrogen, USA) were used at a 1:2000 dilution. Mouse anti-actin (Sigma, USA) was used at a 1:10,000 dilution. Rabbit anti-NPMpT199, cyclin B and XIAP (Cell Signalling Technology, USA) were used at a 1:1000 dilution. Rabbit anti-cyclin A, cdk4 and cdk2 (Santa cruz, USA), anti-Flag (Cell Signalling Technology, USA) and anti-NPMpS125 (Abcam, UK) were used at a 1:2000 dilution. Mouse anti-PP1β and anti-c-myc (Santa Cruz, USA) were used at a 1:2000 dilution. Rabbit anti-NPMpS4 (Cell Signalling Technology, USA) was used at a 1:5000 dilution. Rabbit anti-NPMp234/7 (Abcam, UK) was used at a 1:1000 dilution. The following day, blots were washed three times (5 min each) in TBST and incubated with horse-radish peroxidase (HRP) conjugated goat anti-mouse or anti-rabbit secondary antibodies (Santa Cruz Biotechnology, USA). Protein bands were visualized with Pierce Biotechnology SuperSignal West Pico or Femto chemiluminescent substrate following the manufacturer's recommendations.

## 2.7 Transfection

Cells were seeded into wells of 6-well plates containing autoclaved microscope cover slips such that they were 70-80% confluent at the time of transfection. 5μg of DNA was transfected into each well using 10μl of Lipofectamine 2000 (Invitrogen, USA) per well in serum-free RPMI. The next day, cells were checked for fluorescence.

For cdk2 knockdown, 100nM of control or cdk2 siRNA (Santa Cruz) was transfected with 10μl of Dharmafect (GE healthcare) for 48 hours.

## 2.8 Immunofluorescence

HEK293T cells were grown directly on glass coverslips while AML2/3 cells were cytospun onto microscope slides at 600 rpm for 5 mins.

For NPMpT199 and PP1β staining, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 mins, followed by a wash with PBS and 5 mins permeabilization in digitonin (50μg/ml in PBS) and quenched for 5 mins with 50mM $NH_4Cl$ in PBS. For γ-tubulin or cdk2 staining, cells were fixed/permeabilised in 50:50 ice-cold acetone:methanol for 30 mins and then washed once with PBS before proceeding to blocking. Blocking buffer consists of 5% goat serum and 2% horse serum in PBS for 1 hr at room temperature. Samples were incubated with γ-tubulin primary antibody (T3559, Sigma) at 1:300 dilution factor, cdk2 primary antibody (sc-748, Santa Cruz) at 1:50 dilution factor, PP1β primary antibody (A1088, Abclonal) or phospho-$NPM^{T199}$ primary antibody (3541, Cell signaling) at 1:500 dilution factor in blocking buffer at room temperature for 1 hr. Alexa Fluor-conjugated anti-mouse or anti-rabbit immunoglobulins (Life Technologies) were used as secondary antibodies (1:1000 in blocking buffer for 1 hour at room temperature) and cell nuclei were visualized with DAPI. Fluorescent images were taken using BX60 microscope (Olympus) and

processed using Image J. 100 cells were counted per experiment for centrosome counting.

## *2.9 Cell cycle analysis*

Cells were fixed in 1ml of ice-cold 70% ethanol for at least 3 hours then washed once with PBS and resuspended in Millipore Muse cell cycle reagent (MCH100106, Merck Millipore) as per the manufacturer's instructions. 10,000 events per sample was measured in the Muse Cell Analyzer (0500-3115, Merck Millipore). Experiments were conducted in triplicate and two-sample unequal variance T-test was used to calculate p-value.

## *2.10 Site-directed mutagenesis*

The primers used for site-directed mutagenesis are listed in Table 1.

**Table 1 Primers used for site-directed mutagenesis.**

| | | |
|---|---|---|
| NPMcT199A | Forward | 5'-GTGAAGAAATCTATACGAGATGCCCCAGCCAAAAATGCACAAAAG-3' |
| | Reverse | 5'-CTTTTGTGCATTTTTGGCTGGGGCATCTCGTATAGATTTCTTCAC-3' |
| NPMcS4A | Forward | 5'-CGAATTCTATGGAAGATGCCATGGACATGGACATGAGC 3' |
| | Reverse | 5'-CTCATGTCCATGTCCATGGCATCTTCCATAGAATTCGA 3' |

pAcGFP-NPMc plasmid was used as a template along with 1x *pfu* reaction buffer, 200µM dNTP mix, 0.8µM each of forward and reverse primers and 2µl of *pfu* turbo polymerase (Stratagene, USA) in a total volume of 50µl. Initial denaturation was run at 95˚C for 5min followed by 25 cycles of 95˚C for 30secs, 48˚C for 45secs and 72˚C for 2min. Final extension was carried out for 5min at 72˚C Sequence change was confirmed by sequencing.

### *2.11 Colourimetric caspase activity assay*

Cells were treated with 250nM of staurosporine for 24 hours and lysed in S100 buffer as described above. 50µg of cell lysate was incubated in the reaction buffer with 200µM of LEHD-p*NA* substrate (for caspase 9) or DEVD- p*NA* substrate (for caspase 3) according to the manufacturer's instructions (BioVision, USA). Absorbance at 405nm was read at 2 minute intervals for 16 hours.

### *2.12 Phosphorylated H2A.X assay*

Phosphorylated H2A.X was measured using Muse® Cell Analyser (0500-3115, Merck Millipore) after staining with Muse® H2A.X Activation Dual Detection Kit (MCH200101, Merck Millipore) which contains anti-phospho-Histone H2A.X (Ser139)-Alexa Fluor®555 and anti-Histone H2A.X-PECy5 conjugated antibodies.

### *2.13 Cell death assay*

Live and apoptotic cells were analysed via annexin V and PI staining using Muse® Annexin V and Dead Cell Kit (MCH100105, Merck Millipore) according to the manufacturer‘s protocol. Samples were processed using Muse® Cell Analyser (0500-3115, Merck Millipore).

### *2.14 Statistics*

All statistics were computed in Microsoft Excel. All error bars in figures represent standard error. P-value was calculated based on student's T-test with unequal variance.

# 3 XIAP regulation and expression in NPMc expressing cells

## *3.1 Introduction*

The cytoplasmic localisation of NPM has previously been shown to inhibit caspases 6 and 8 of the extrinsic cell death pathway leading to mitigated TRAIL-triggered apoptosis and inhibited myeloid differentiation (Leong et al. 2010). But what of the intrinsic (mitochondrial) cell death pathway involving caspases 9, 3 and 7?

XIAP is a known inhibitor of caspases 9, 3 and 7 (LaCasse 2013). Clinical data also show that patients with high levels of XIAP confer poor response to chemotherapy (Ibrahim et al. 2012) leading to poorer prognosis (Sung et al. 2009). It is no surprise, therefore, that XIAP is being targeted by therapeutic drugs and there are studies evaluating their effectiveness in both AML (Schimmer et al. 2011) as well as solid cancers (Mahadevan et al. 2013).

### 3.1.1 Rationale

Previous work in the lab has shown that NPMc overexpression causes increased X-linked inhibitor of apoptosis (XIAP) protein levels and that this occurs by the transcription factor Sp1 but not other pathways such as RIP, JNK or NF-κB (Loo 2009; Wong 2010). XIAP is the most potent inhibitor of caspase and also the most widely expressed IAP (Obexer and Ausserlechner 2014). It has also been found that the NPMc-expressing cell line OCI-AML3 expresses XIAP at higher levels compared to other leukaemic cell lines, HL-60, KG-1 and K562 (Carter et al. 2003). In addition, XIAP expression levels are more strongly correlated with prognosis than other members of the IAP family such as Survivin (Tamm et al. 2000).

Further work in the lab found that HEK293T cells overexpressing GFP-tagged NPMc (GFP-NPMc) show an upregulation of XIAP compared to GFP or GFP-tagged wild

type NPM (GFP-wtNPM). In addition, chromatin immunoprecipitation showed that binding of the transcription factor Sp1 to the XIAP promoter was higher in GFP-NPMc overexpressing cells than GFP-wtNPM and GFP overexpressing cells. However, Sp1 expression levels did not correlate with promoter binding. Therefore, it is more likely that post-translational modification/s and/or protein-protein interactions are responsible for regulating Sp1 promoter binding specificity and activity.

As a transcription factor, Sp1 is well documented to be regulated by post-translational modifications (Li et al., 2004; Tan & Khachigian, 2009). For example, phosphorylation by cdk2 complexed with and activated by cyclin A increased DNA binding and transcription of a reporter gene; furthermore, overexpression of cyclin A increased mRNA expression of the reporter gene (Fojas de Borja et al., 2001). Cdk2 is already known to be linked to XIAP expression; its inhibition with the drug GW8510 decreased both XIAP mRNA and protein levels without affecting other anti-apoptotic proteins such as Bax, Bak or Bcl2. This downregulation of XIAP was accompanied by increased apoptosis in non-small cell lung cancer cells (Dong et al. 2006). Since Sp1 is a known transcription factor of XIAP (Lee et al. 2006) and can be regulated by cdk2 phosphorylation, it is reasonable to postulate that the downregulation of XIAP reported by Dong et al may be caused by decreased phosphorylation by cdk2 and hence promoter binding of Sp1.

As previously mentioned, expression of Sp1 responsive genes is correlated to cyclin A levels (Fojas de Borja et al. 2001) and cdk2, like all cdks, requires cooperation with a cyclin in order to function. Is cyclin A upregulated in NPMc cells? And if so, how so?

As a multi-functional protein, NPM can bind to many proteins and its delocalisation can therefore cause the export of these proteins into the cytoplasm. One such protein is fbw7γ, a ubiquitin ligase and hence, degradation trigger for oncogenes such as cyclin

E, myc, jun and notch (Welcker and Clurman 2008). It's delocalisation into the cytoplasm by NPMc induces its degradation thereby stabilising myc and increasing expression of myc's target genes (Bonetti et al. 2008). Therefore, we postulate that this stabilisation of myc in NPMc-expressing cells leads to increased expression of cyclin A and hence increased Sp1 binding and XIAP expression. This chapter, therefore, aims to study the mechanism by which XIAP may be regulated in NPMc-expressing cells.

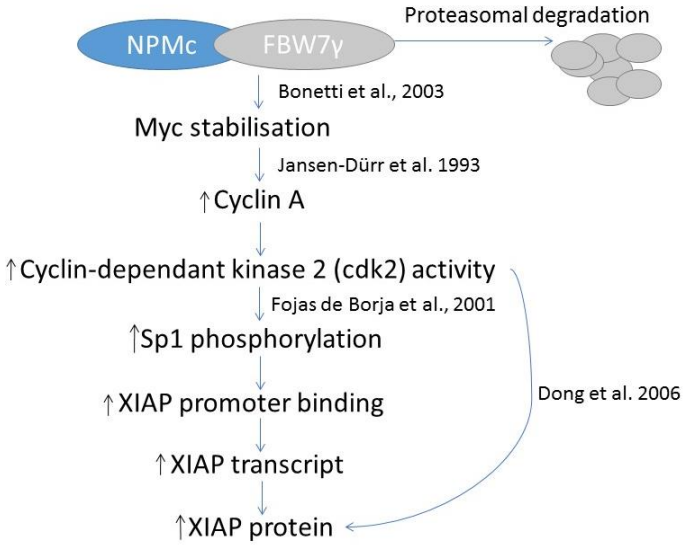


**Figure 3.1 Hypothesis of XIAP regulation in NPMc-expressing cells.**

## *3.2 Results*

To facilitate this study, two cell line systems were used: haematopoietic myeloid progenitor cell lines OCI-AML2 and OCI-AML3; and human embryonic kidney with T-antigen (HEK293T) stable cell lines overexpressing GFP, GFP-wtNPM and GFP-NPMc. The former system is more reflective of the disease but the disadvantages are that OCI-AML2 and OCI-AML3 (henceforth referred to as AML2 and AML3, respectively) are not isogenic as they are isolated from different patients and are also more difficult to maintain in cell culture, having the propensity to differentiate if not properly passaged. For this reason, the HEK293T stable cell lines, previously

established in the lab (Chan 2010) via lentiviral transfection, are used to complement AML2/3.

### 3.2.1 NPM binds to Fbw7γ which regulates myc stability

To corroborate the findings of Bonetti et al. (Bonetti et al. 2008), a GST pulldown was used on lysates of HEK293T cells transfected with flag-tagged Fbw7γ. Consistent with their report, GST-NPM was able to pull down flag-fbw7γ as the latter was depleted in the supernatant (Figure 3.2, right most lane), indicating that flag-fbw7γ had been removed from the cell lysate by NPMc. Deletion fragments of NPM show that the N-terminal fragments are unable to pull down flag-fbw7γ more than the GST control while the C-terminal 187-294 residues were able to pull down flag-fbw7γ to a similar degree compared to the larger 118-294 amino acid fragment. This indicates that the last 108 amino acids are sufficient to bind flag-fbw7γ and does not require structural support from other domains.

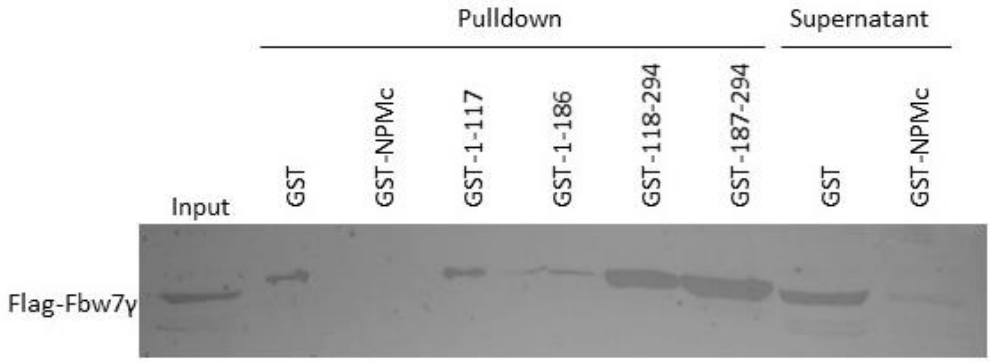


**Figure 3.2 NPM's C-terminal binds to fbw7γ.**
GST pull-down of Fbw7γ with NPM fragments as labelled. Blot probed with anti-flag.

It is unclear whether the C-terminal 112 amino acids of NPMc can bind to fbw7γ in a similar way to the C-terminal 108 residues of wtNPM. NPMc's last 50 residues have been shown to be more unfolded than wtNPM (Grummitt et al. 2008; Scaloni et al. 2010) so fbw7γ binding may require full-length NPMc unlike wtNPM which can still bind fbw7γ with only the last 108 amino acids. A GST-NPMc truncation mutant would

be needed to determine whether NPMc's C-terminal frame-shift mutation, which only changes the last 7 residues, could affect its ability to bind fbw7γ.

Consistent with the hypothesis and previous findings (Bonetti et al. 2008), the stability of myc protein is dependent on NPMc localisation. Treatment of cells with leptomycin B, an inhibitor of nuclear export resulted in a decrease of myc protein levels in NPMc expressing HEK293T cells as well as AML3 but not GFP, GFP-wtNPM controls or AML2. Reverse transcription polymerase chain reaction (RT-PCR) of myc mRNA showed that the decrease in myc protein levels was not due to decreased myc gene transcription as cDNA levels did not show a corresponding decrease but in fact increased (Figure 3.3).

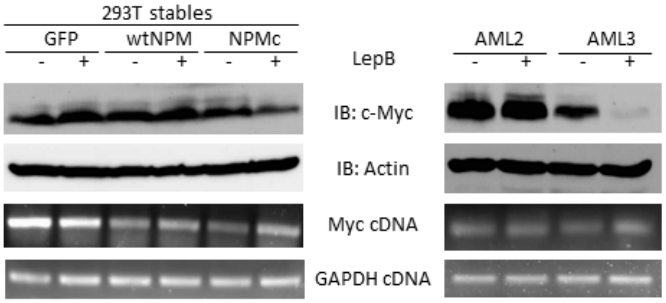


**Figure 3.3 Decrease of myc upon leptomycin B (LepB) treatment is not due to decrease in mRNA levels.**
Cells were treated with 5nM of leptomycin B for 4 hours then run on Western blot or had their RNA extracted and then RT-PCRed for the indicated gene. IB, immunoblot.

The stability of myc was also assayed using cycloheximide, an inhibitor of protein translation. In NPMc-overexpressing HEK293T cells, myc was more stable than in GFP or GFP-wtNPM overexpressing counterparts. However, in the myeoblastic AML2 and 3, myc was less stable in the NPMc expressing AML3 than in AML2, being degraded to a ~43kDa fragment (Figure 3.4). This 43kDa fragment was still detectable by the myc antibody (9E10) raised against residues 408-439 so any cleavage would likely occur at the N-terminus leaving the DNA binding domain at the C-terminus unaffected.

Therefore the transactivating ability of the 43kDa fragment should not be negated. This fragment was not seen in the HEK293T cells.

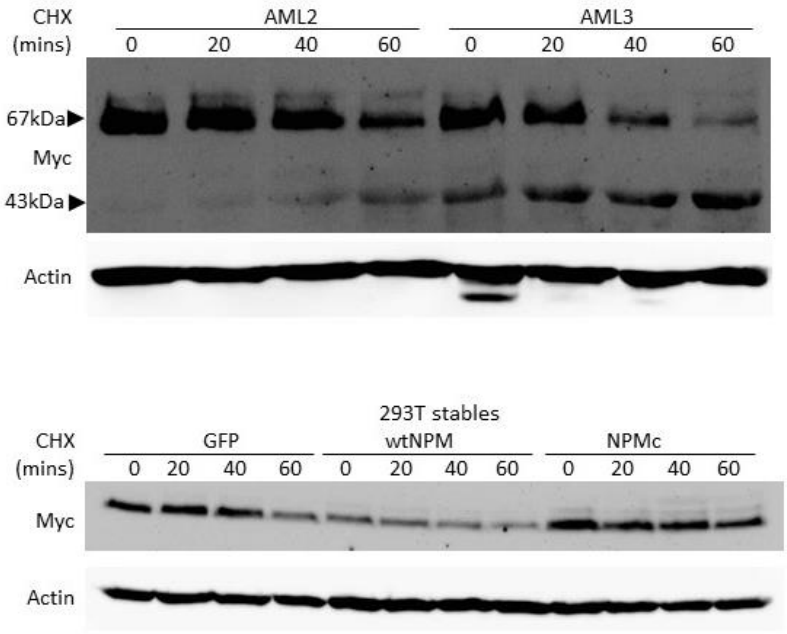


**Figure 3.4 Cycloheximide (CHX) chase assay showing that myc is more persistent in NPMc-expressing 293T than GFP or wtNPM counterparts.**
Contrary to hypothesis, myc in AML3 is not more stable than AML2. However, note the ~43kDa band which still retains the C-terminal (detected by 9E10 antibody) DNA binding domain.

### 3.2.2 XIAP protein level is inconsistent with mRNA expression and does not decrease upon cdk2 inhibition

Next, the protein levels of cyclin A were compared. Contrary to the hypothesis, there is no significant difference between the GFP, GFP-wtNPM and GFP-NPMc overexpressing HEK293T nor AML2 and AML3. However, RT-PCR of XIAP was still consistent with the hypothesis of the NPMc expressing cells having higher expression than AML2 or GFP and GFP-wtNPM expressing HEK293T.

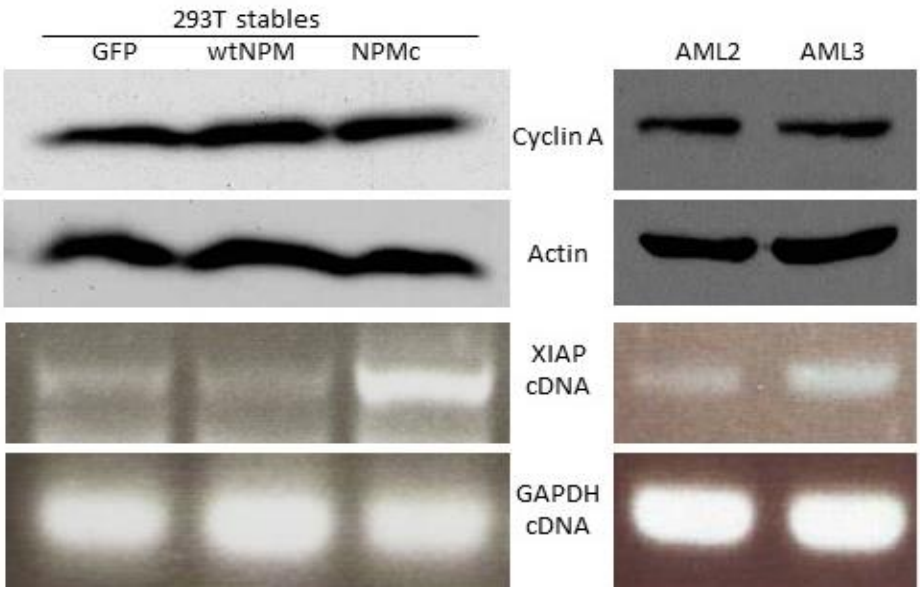


**Figure 3.5 XIAP mRNA levels are higher in NPMc-expressing cells but Cyclin A levels do not differ significantly.**
Actin was used as a loading control for protein and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for RT-PCR.

As reported by Dong et al (Dong et al. 2006), treatment with cdk2 inhibitor GW8510 decreased XIAP levels in non-small cell lung cancer cells. To verify that GW8510 does indeed inhibit cdk2, cells were treated with 5 and 10μM GW8510 in DMSO and the effect on cell cycle compared to controls. As expected, the drug decreased the number of cells in G0/G1 phase with a corresponding increase in the number of cells in S and G2/M phase as cdk2 inhibition would prevent the proper completion of S/G2 and attenuate the progression through M phase (Figure 3.6). Interestingly, in both AML and HEK293T, the cells expressing NPMc had more cells in G0/G1 than those with wtNPM. There was little significant difference between the 5 and 10μM treatments so 5μM was used for subsequent experiments.

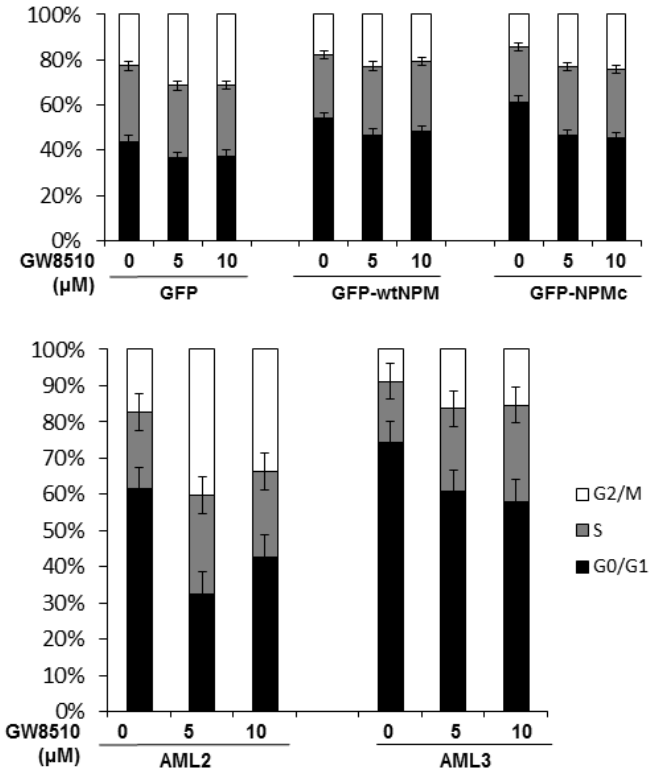


**Figure 3.6 GW8510 treatment inhibits mitotic progression causing cells to build up in S and G2/M phase.**
Cells were treated with stated amounts of drug overnight or the solvent DMSO as control then fixed and stained with potassium iodide before analyses by low cytometry. Upper panel, HEK293T stably expressing GFP, GFP-wtNPM or GFP-NPMc; Lower panel, hematopoietic cell lines OCI-AML2 (AML2) and OCI-AML3 (AML3). Results are representative of three independent experiments. Error bars represent standard error.

Although cdk2 inhibition produced the expected effect on cell cycle, it did not replicate the results reported by Dong et al. There was also no observable decrease in XIAP levels upon GW8510 treatment in all cells except for the GFP overexpressing HEK293T (Figure 3.7). XIAP levels also seemed unperturbed in both AML2 and 3 upon treatment. However, XIAP levels also were lower in NPMc expressing cells, contrary to the mRNA results. Although AML3 displayed higher XIAP protein levels than AML2, NPMc-overexpressing 293T did not display this same phenotype. Therefore it is unlikely that NPMc in itself can upregulate XIAP expression.

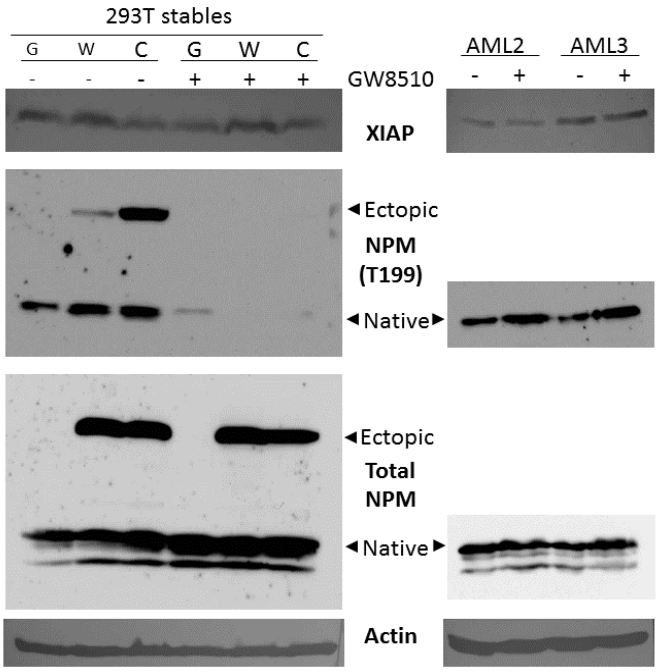


**Figure 3.7 XIAP levels are not increased in NPMc expressing cells and do not decrease upon GW8510 treatment.**
Cells were treated for 16 hours with 5μM of cyclin-dependant kinase 2 (cdk2) inhibitor GW8510 which abolished the phosphorylation of both endogenous as well as GFP tagged exogenous NPM at threonine 199 (T199). NPMc(C)-, GFP (G) and wtNPM (W) overexpressing cells.

As further verification that cdk2 was indeed being inhibited by GW8510, blots were also probed for phosphorylated NPM (threonine 199, T199) known to be phosphorylated by cdk2 as a permissive precursor to centrosome duplication (Tokuyama et al., 2001). GW8510 was able to decrease phosphorylation in the HEK293T cells but not AML2 and AML3. Interestingly, the NPMc expressing cells showed a higher level of T199 phosphorylation compared to AML2 and GFP or GFP-wtNPM overexpressing HEK293T.

### 3.2.3 NPMc-expressing cells have greater caspase 3 and 9 activity

To further corroborate the low XIAP levels in the HEK293T stables, the activity of caspases 3 and 9 which XIAP inhibits were measured using a colourimetric assay which uses colourless substrates that turn yellow when cleaved by specific caspases. The amount of yellow substrate can then be quantified by the absorbance of 405nm light

and is positively correlated with caspase activity. Cells were treated with the initiator of apoptosis staurosporine known to activate caspases by compromising mitochondrial integrity resulting in the release of cytochrome c, caspase 3 and caspase 9 (Chae et al. 2000; Belmokhtar et al. 2001; Fiorucci et al. 2002; Zhang et al. 2004b). Cells expressing NPMc had higher caspase 3 and 9 activity for both staurosporine and control treatments with the solvent DMSO indicating that NPMc did not provide any protection against the intrinsic cell death induced by staurosporine but instead increased caspase 3 and 9 activity (Figure 3.8). The lower XIAP level of the NPMc-expressing cells is therefore linked to a decreased ability to inhibit apoptosis.

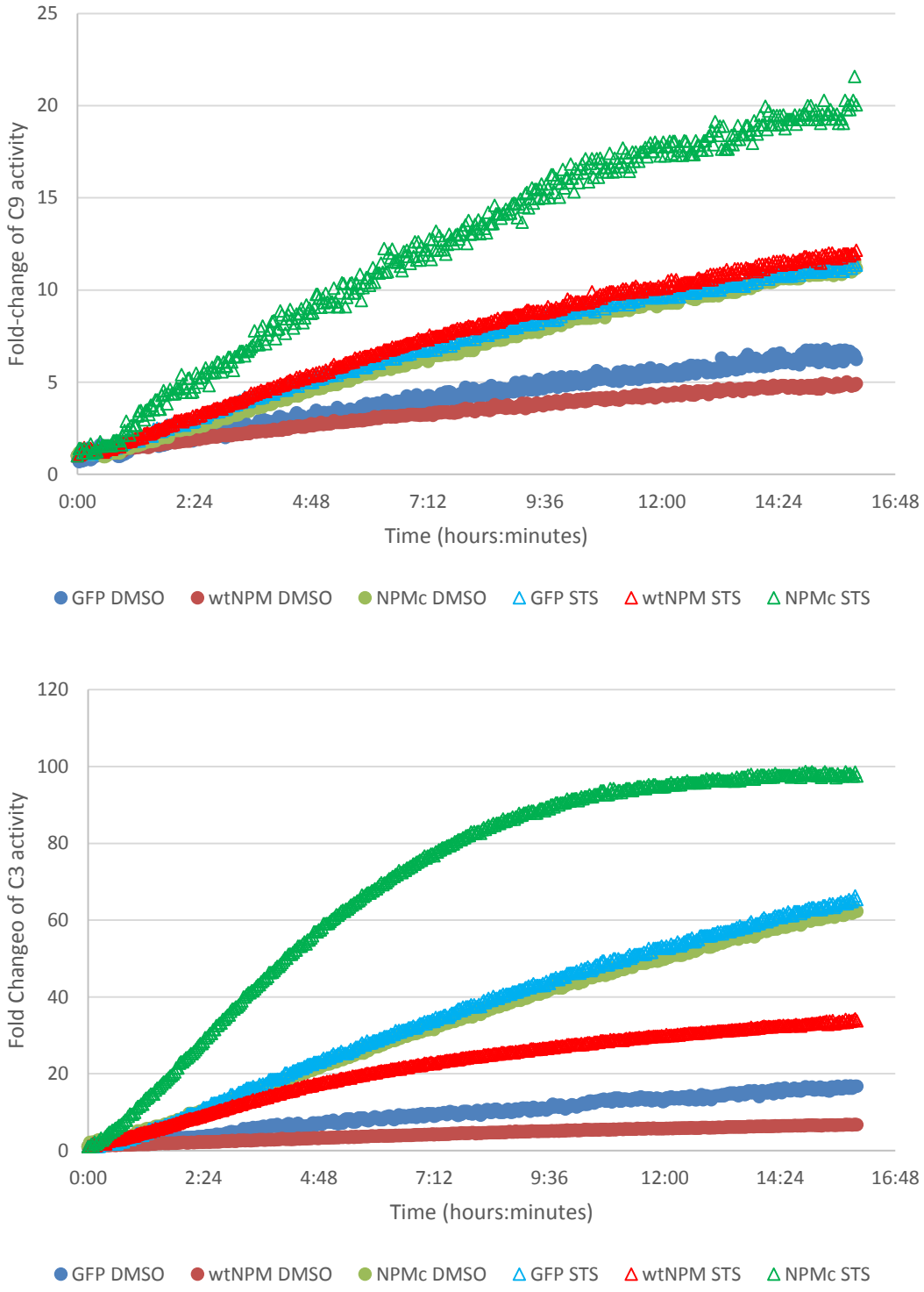


**Figure 3.8 NPMc-expressing cells have higher caspase 3 and 9 activity than GFP or wtNPM counterparts.**
HEK293T cell lines stably expressing GFP, GFP-wtNPM (wtNPM) or GFP-NPMc (NPMc) were treated with 250nM of staurosporine (STS) for 24 hours and lysed in S100. 50µg of cell lysate was incubated in the reaction buffer with 200µM of LEHD-pNA substrate (for caspase 9, upper panel) or DEVD- pNA substrate (for caspase 3, lower panel) according to the manufacturer's instructions (BioVision, USA). Absorbance at 405nm was read at 2 minute intervals for 16 hours.

## *3.3 Discussion*

### 3.3.1 The complexities of XIAP regulation.

Although increased XIAP expression was observed in the NPMc-expressing AML3 cells compared to wtNPM-expressing AML2, NPMc-expressing HEK293T cells had decreased XIAP levels compared to GFP or wtNPM counterparts. Therefore it is unlikely that NPMc is directly involved in XIAP regulation. Indeed, another study have also found XIAP mRNA to be inconsistent with protein levels (Tamm et al. 2000).

We were unable to replicate the results by Dong et al with regards to the downregulation of XIAP in response to the cdk2 inhibitor GW8510 perhaps because they were cell specific; although there has been precedence for the downregulation of XIAP in response to another small molecule cdk inhibitor, flavopiridol, in breast cancer cells (Wittmann et al. 2003).

Even though XIAP may be successfully transcribed and translated, it does not guarantee protection against intrinsic cell death. XIAP can be inhibited by Smac/DIABLO which blocks its binding ability to caspases (Obexer and Ausserlechner 2014). Similar to NPM's role in caspase 6 and 8 inhibition, XIAP must also be in the cytoplasm to inhibit caspases; relocalisation into the nucleus by interaction with XIAP –associated factor 1 leads to decreased caspase 3 activity and cell death (Liston et al. 2001; Russell et al. 2008). XIAP may also be cleaved by the very caspases it is meant to inhibit: XIAP's inhibition of intrinsic cell death can be overcome by the activation of caspase 3 through the extrinsic pathway, cleaving and inactivating XIAP (Deveraux et al. 1999). Finally, XIAP's degradation can be accelerated by ubiquitin ligases in addition to its own autoubiquitination ability (Hiramatsu et al. 2014). Phosphorylation by Akt on serine 87 inhibits this, stabilising XIAP and increasing resistance to cell death (Dan et al. 2004).

XIAP in AML3 is already known to be regulated by PI3 kinase and to a lesser extent MEK kinase (Carter et al. 2003) and while the status of these pathways in AML2 is not known, it is an interesting possibility to explore in future. Therefore the higher expression of XIAP in AML3 cells compared to AML2 should not be taken as solid evidence for the resistance to cell death or promotion of cell survivability as NPMc+ AML have favourable prognoses in clinical settings.

### 3.3.2 The non-isogenicity of AML2/3.

Another limitation is that AML2 and AML3 are not isogenic despite both being classed under the same group according to the French-American-British classification system (Table 2). The former was isolated from the peripheral blood of a 65-year-old male while the latter was likewise isolated from a 57-year-old male. Both cell lines have hyperdiploid karyotype but AML2 has much more cytogenetic abnormalities than AML3 (Wang et al. 1989). On a molecular level, although both AML2 and AML3 have wild-type p53, the former overexpresses Mdm4 which binds to p53, sequestering and stabilising it in the cytoplasm. AML3 therefore has less stable p53 than AML2 which may affect apoptotic response (Tan et al. 2014). AML3 also has a reduced ability to repair DNA lesions compared to AML2 as NPMc binds to the repair polymerase, DNA polη, exporting it, resulting in its degradation (Ziv et al. 2014). Contradictorily, AML3 is more sensitive than AML2 to chemotherapeutics targeting DNA damage such as doxorubicin and etoposide (Lew et al. 2011). DNA methylation states may also be different between AML2 and AML3. Both cell lines have mutations in the methyltransferase domain of DNA methyltransferase 3A but AML2's is towards the N-terminus (R635) while AML3's is at the C-terminus (R882C). These may affect the enzyme's activity differently and hence give the two cell lines different epigenetic

profiles (Tiacci et al. 2012). NPMc+ AML is already known to have a distinct DNA methylation signature (Figueroa et al. 2010). Therefore the upregulation of XIAP in AML3 may be due to differences in gene regulation unrelated to NPM status.

AML2 and 3 also displayed different c-Myc degradation profiles; the former retaining full-length myc for a longer period after translation inhibition while myc in AML3 was quickly cleaved by an unknown protease to 43kDa (Figure 3.4). Shortened variants of myc have been known to be biologically active. Myc-nick is cleaved at lysine 298 by calpains, recruits the acetyltransferase GCN5 to tubulins, increases α-tubulin acetylation and promotes myoblast differentiation (Conacci-Sorrell et al. 2010). MycS is produced from an alternative start codon and is missing ~100 amino acids of the N-terminus. Although this deletes one of the four Myc boxes in the transactivation domain, MycS still retains most of the function of Myc and can rescue phenotypes of myc null cells such as enhanced cell proliferation and anchorage-independent growth (Xiao et al. 1998). Therefore the biological activity of the 43kDa fragment should not be dismissed and could potentially lead to a new research direction for myc.

**Table 2 Peculiarities of AML2 versus AML3.**
Der, derived from; del, deletion; inv, inversion; t, translocation; dup, duplication; ins, insertion; CD, cluster of differentiation marker; HLA-DR, human leukocyte antigen-D related; ANLL, Acute nonlymphocytic leukaemia; RB, retinoblastoma; FAB, French-American-British category .

| | OCI-AML2 | OCI-AML3 |
|---|---|---|
| Morphology | single, round to oval cells in suspension | single and clumps, mostly round cells growing in suspension |
| Doubling time | about 30-50 hours | about 35-40 hours |
| Immunology | CD3-, CD4+, CD13+, CD14-, CD15+, CD19-, CD33+, CD34-, CD68+, HLA-DR+ | CD3-, CD4+, CD13+ CD14-, CD15+, CD19-, CD34+, CD68+, HLA-DR- |
| Cytogenetics | human hyperdiploid karyotype with 3.8% polyploidy - 48(43-49)<2n>XY,+6,+8, der(1)inv(1)(p36q31)t(1;6)(q13;p12), der(2)t(2;17)(p23;q24.1)del(2)(q14.2q36), der(3)t(1;3)(p36;p25), ins(3;2)(q21;q14.2q36), t(5;8)(q11.2;q24), der(6)t(1;6)(q31;p12)t(3;6)(q26;q24), inv(12)(p13.3q13.2), t(13;14)(q32/33;q24.2), der(17)t(2;17)(p23;q24.1) sideline with +der(5) - carries apparent variant translocations involving several ANLL breakpoints: 1p36, 3q21, 3q26 (megakaryocytic abnormalities), 12p13, 17q24 (FAB-M4) | human hyperdiploid karyotype - 48(45-50)<2n>X/XY, +1, +5, +8, der(1)t(1;18)(p11;q11), ins(5p), del(13)(q13q21), dup(17)(q21q25) - sideline with r(Y)x1-2 - hemizygous for RB1 |

### 3.3.3 A new direction: The elevation of T199 phosphorylation.

Perhaps the most interesting find in this chapter is the elevation of the centrosome-related phosphorylation site threonine 199 (T199) in NPMc because it occurred in both the AML system and the HEK293T stable cell lines, implying that it is unlikely to be caused by differences in genetic backgrounds.

Phosphorylation was decreased upon treatment with cdk2 inhibitor GW8510 in HEK293T but not AML cells. This could be due to a lack of cell permeability in AML or other cdks compensating for the downregulation of cdk2 activity caused by GW8510.

It is known that there exists a certain level of redundancy among both cyclins and cdks (Satyanarayana and Kaldis 2009). Cdk2$^{-/-}$ mouse are viable albeit meiosis is affected leading to sterility (Ortega et al. 2003). So it is possible that other cdks (e.g. cdk4 and/or cdk1) could compensatorily phosphorylate T199 although why this occurs in AML but not HEK293T is unknown. It could be that AML2 and 3, originating from cancer cells, have a greater propensity for non-canonical cyclin/cdk activity that would support cell proliferation if any particular cyclin/cdk were to be somehow incapacitated during the tumour transformation process. Whereas HEK293T is derived from an otherwise healthy aborted foetus (van der Eb 2001) and would have less impetus to stray from orthodox cyclin/cdk activity.

Nevertheless, there is a lack of literature on the topic of NPMc and centrosomes. While the role of wtNPM in centrosome licensing has been known for some time (Okuda et al. 2000), it seems that the pathological displacement of NPM into the same subcellular compartment as the centrosome (cytoplasm, as opposed to being physically separated by the nuclear envelope) has received little attention even though it would stand to reason that such a delocalisation could be part of leukaemogenesis especially in light of the correlation between NPMc and normal karyotype (85%, Estey 2010) which is in part maintained by faithful centrosome duplication. The rest of this thesis therefore begins to fill this gap.

# 4 Causes of increased NPMc phosphorylation

## *4.1 Introduction*

During the cell cycle, NPM is phosphorylated by cyclin dependant kinase 2 on threonine 199 (T199) which causes the dissociation of NPM away from the centrosome and permits duplication to occur. Mutation of the phosphorylation site to non-phosphorylatable alanine prevents phosphorylation and hence centrosome duplication, thus increasing the number of cells with one centrosome and decreasing the number of cells with two centrosomes (Tokuyama et al., 2001). However, a more recent study, in which endogenous NPM was knocked out in $Arf^{-/-}/p53^{-/-}/Mdm2^{-/-}$ triple knockout mouse embryonic fibroblasts, showed that infection with a non-phosphorylatable NPM mutant at the corresponding mouse phosphorylation site (T198) was able to rescue centrosome numbers to a similar degree as those in infected with wild-type NPM (Brady et al. 2009). The authors postulated that NPM's suggested role as a centrosome duplication inhibitor may be a downstream effect of its ribosome biogenesis/protein production role since the non-phosphorylatable T198A NPM mutant was able to rescue ribosome levels to NPM knockouts. However an alternate possibility is that other centrosomal factors were able to license duplication or the non-phosphorylatable NPM could be sequestered away from the centrosome by other cytoplasmic factors hence allowing duplication. NPM, being a multi-functional protein, has multiple binding partners so there is no shortage of candidates in this regard.

In NPMc+ AML, the cytoplasmic load of NPM increases and the results in this chapter aim to eliminate other possible causes of elevated NPMcT199 phosphorylation, further pointing to aberrant cytoplasmic localisation as the chief cause.

## *4.2 Results*

### 4.2.1 Increased phosphorylation is not dependent on kinase levels, activation or localization

As mentioned in the previous chapter, we suspected that there might be compensatory phosphorylation by other kinases (whether cyclin-dependant or not) occurring in the AML system. To explore this possibility, the HEK293T stable cell model was chosen for its isogenicity, ease of culture and transfectability which enable simplify experiments. To verify that cdk2 was the main kinase in the HEK293T system, cells were transfected with 100nM of cdk2 siRNA for 48 hours and phosphorylation levels of NPM detected on Western blot.

Knockdown of cdk2 greatly reduced phosphorylation confirming previous literature that cdk2 is the predominant kinase for T199 phosphorylation (Adon et al. 2010). NPMc's elevated phosphorylation is not facilitated by increased cdk2 expression as cdk2 levels did not differ between GFP, wtNPM or NPMc overexpressing cells (Figure 4.1a).

To confirm the elevated phosphorylation of NPMc was not due to indirect effects of transgene insertion, HEK293T cells were transfected with GFP, GFP-wtNPM or GFP-NPMc and treated with cdk2 inhibitor GW8510 (Johnson et al. 2005; Dong et al. 2006; Diaz-Corrales et al. 2008). GFP-NPMc is more phosphorylated than GFP-wtNPM despite similar total NPM (Figure 4.1b). This phosphorylation was greatly diminished upon cdk2 inhibition across all cell types. Neither cyclin E nor cdk2 were elevated in GFP-NPMc overexpressing cells, indicating that the increased phosphorylation of NPMc does not require increased activation or protein levels of cdk2. In fact, cyclin E was expressed at slightly lower levels in GFP-NPMc overexpressing cells despite the elevated phosphorylation of NPMc. Upon GW8510 treatment, cyclin E levels increased

as would be expected because cdk2 is unable to stimulate the progression to mitosis and more cells become arrested and build up in S phase when cyclin E is expressed (Keck et al. 2007).

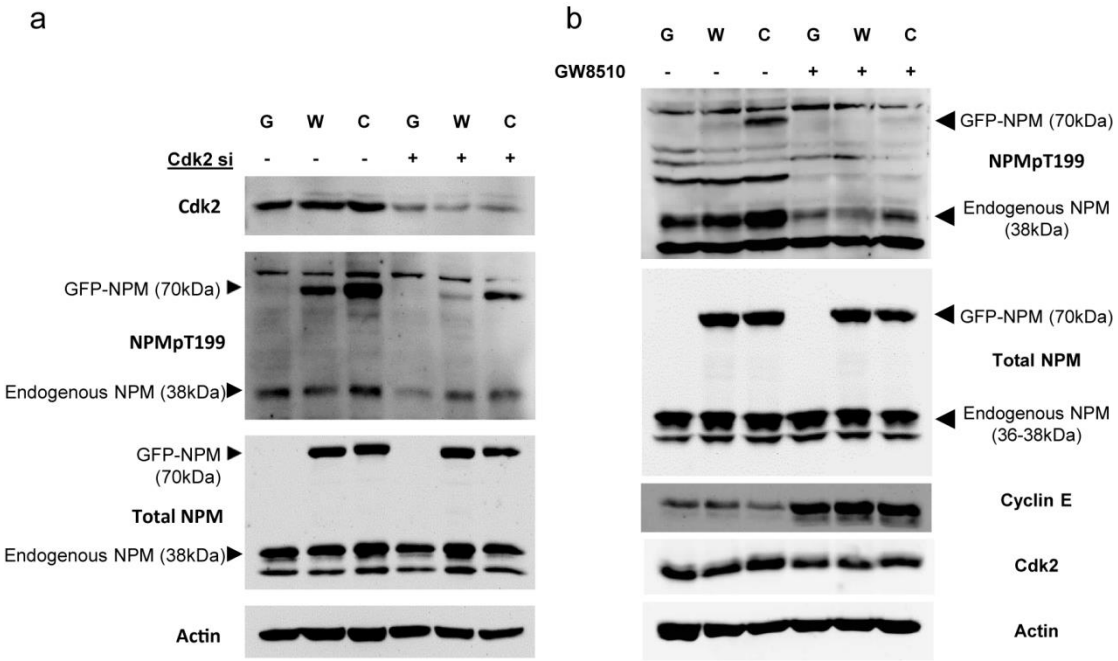


**Figure 4.1 NPMc hyperphosphorylation is not dependent on cdk2 levels or activation**
(a) Western blot shows that knockdown of cdk2 reduces T199 phosphorylation and that NPMc phosphorylation is elevated compared to wtNPM. . HEK293T cell lines stably expressing GFP (G), GFP-wtNPM (W) or GFP-NPMc (C) were transfected with 100nM of cdk2 siRNA for 48hours. (b) Western blot of HEK293T cells transfect with GFP (G), GFP-wtNPM (W) or GFP-NPMc (C) for 24 hours before drug treatment selected cell cycle proteins in response to 5μM GW8510 treatment for 24 hours shows cdk2 inhibition abolished the phosphorylation of both endogenous as well as GFP tagged exogenous NPM at threonine 199 (T199). Adapted from Chan and Lim 2015.

Furthermore, immunofluorescence staining of cdk2 shows that it is not restricted to any subcellular location, neither does its localization differ between wild-type or NPMc-overexpressing cells (Figure 4.2a). However, since the staining with the antibody from Santa Cruz appeared non-specific, immunofluorescence was repeated with another cdk2 antibody from a different source (Abcam, Figure 4.2b). The Abcam antibody showed a more nuclear staining pattern consistent with the manufacturer's datasheet. Regardless, neither antibody displayed any preference for cdk2 in the cytoplasm so the

elevated phosphorylation of NPMc is therefore more likely due to the increased cytoplasmic load of NPM rather than the levels, activation or localization of cdk2.

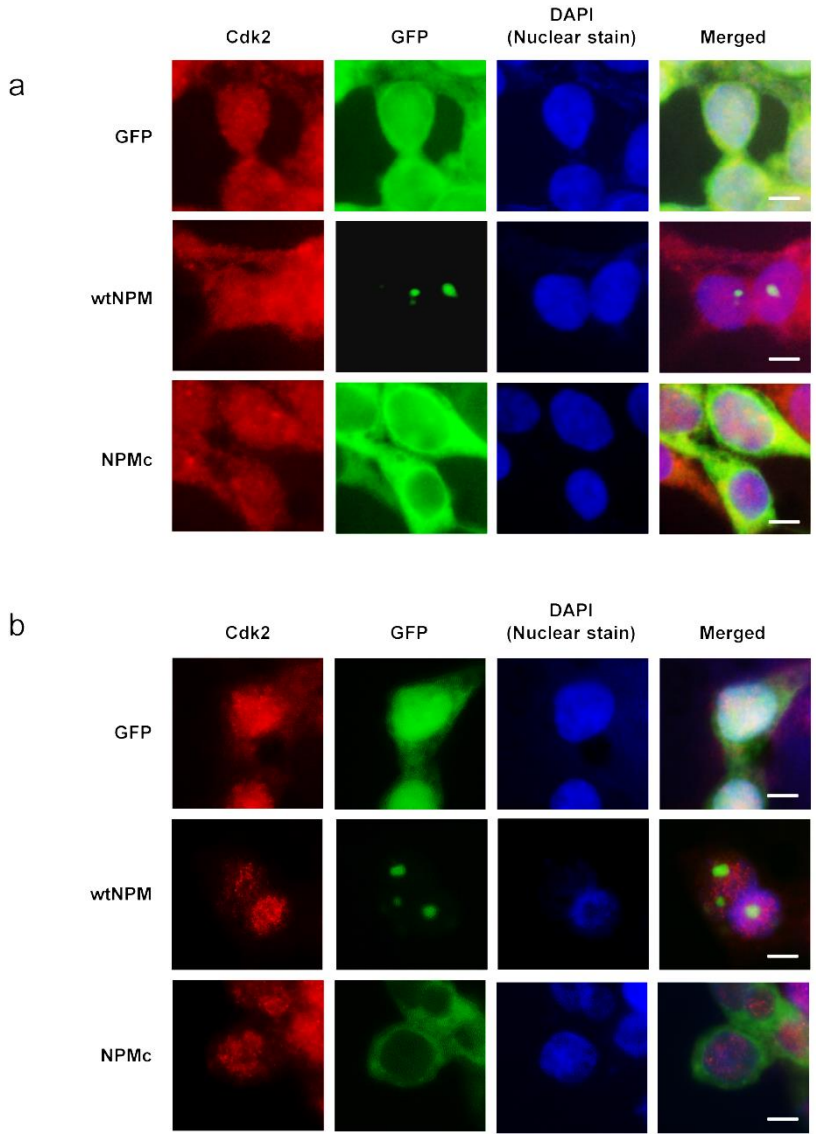


**Figure 4.2 Cdk2 is not preferentially localised to the cytoplasm.**
Immunofluorescence staining of (a) Anti-cdk2 (H-298) from Santa Cruz (sc-748) and (b) Anti-cdk2 from Abcam (ab6538). Bar=20μM. Adapted from Chan and Lim 2015.

As there is a certain level of redundancy among cyclins and cdks (Satyanarayana and Kaldis 2009), the elevated phosphorylation of NPMc may be facilitated by the increased expression or activation or these other kinases. However, a brief panel of other cyclins and cdks (Figure 4.3) show that this is not the case. Levels of Cyclin B and cdk4 are not significantly different from GFP- and wtNPM-expressing controls and cyclin A and

cdk1 are slightly decreased in comparison. In addition, levels of the phosphatase which dephosphorylates T199, protein phosphatase 1 β (Lin et al. 2010), are similar in all three cell types. This further supports the hypothesis that NPM localisation is the main factor in determining T199 phosphorylation and not other extraneous protein interactions.

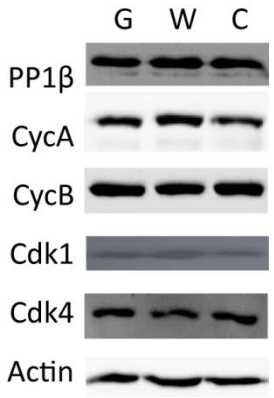


**Figure 4.3 Other cell cycle proteins and phosphatase are not elevated in NPMc-expressing cells.**
Western blot of HEK293T stable cell lines. G, HEK293T stably overexpressing GFP; W, HEK293T stably overexpressing GFP-wtNPM; C, HEK293T stably overexpressing GFP-NPMc. PP1β, protein phosphatase 1 β; CycA, cyclin A; CycB, cyclin B; Cdk, cyclin dependant kinase.

### 4.2.2 NPM phosphorylation is dependant on subcellular localisation

As shown in the previous chapter, NPMc is more phosphorylated than wtNPM and this phosphorylation is abolished upon cdk2 inhibition with the drug GW8510. To further verify this and see whether this phosphorylation is dependent on NPM's localisation, cells were pre-treated with leptomycin B for 1 hour before addition of DMSO or GW8510 and then blotted for phosphorylated NPM. After 1 hour, cells were briefly checked under a light microscope (Figure 4.4) for the relocalisation of NPMc to the nucleus. Inhibition of nuclear export did decrease phosphorylation of GFP-NPMc but not GFP-wtNPM. Leptomycin B also further enhanced the decrease in phosphorylation caused by GW8510's cdk2 inhibition (Figure 4.5).

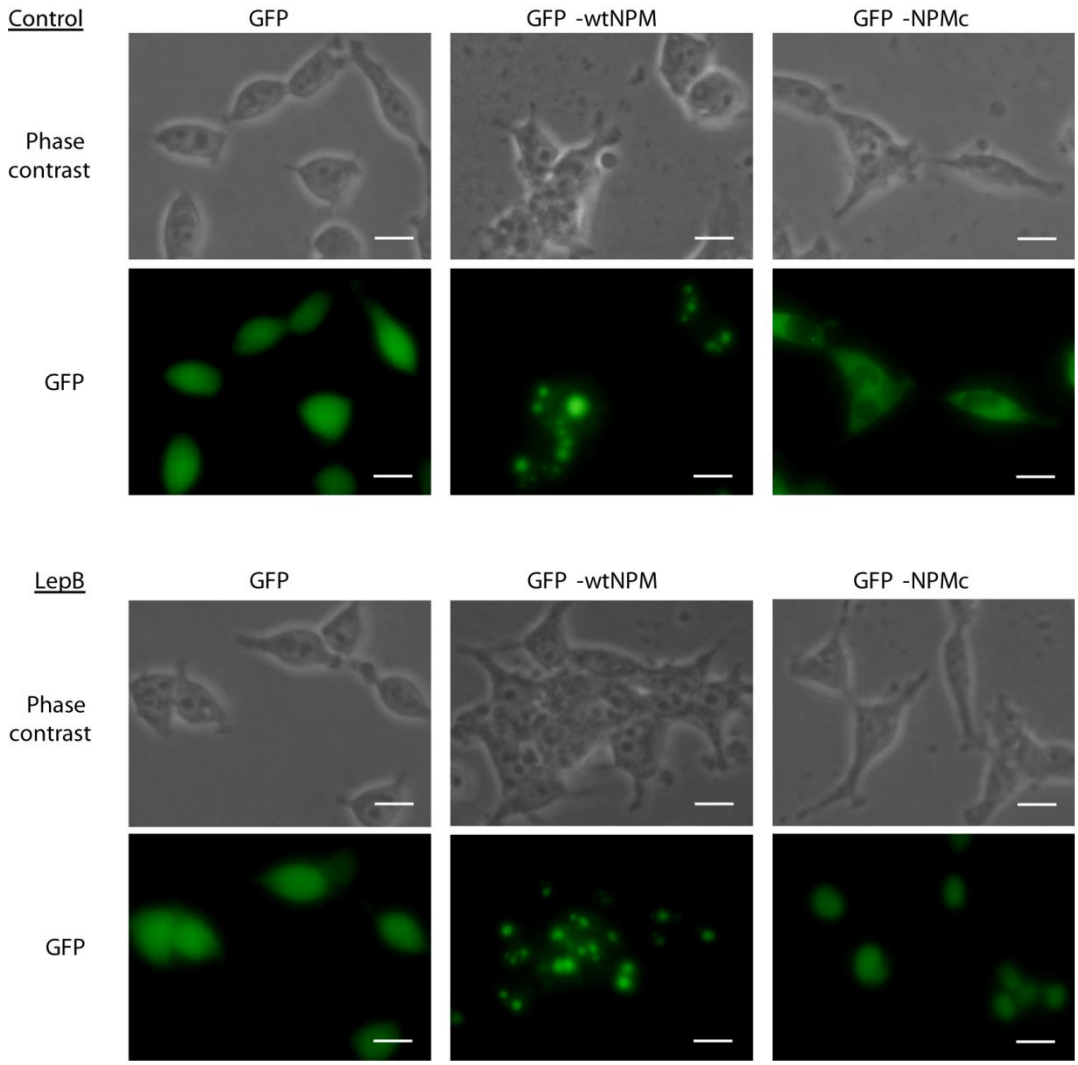


**Figure 4.4 Leptomycin B causes relocalisation on NPMc into the nucleus but does not affect wtNPM or GFP localization.**
HEK293T stably expressing GFP, GFP-wtNPM (wtNPM) or GFP-NPMc (NPMc) were treated with 5nM leptomycin B (lepB, bottom 6 panels) for 24 hours. The localisation of NPMc (bottom right most panel) changes from cytoplasmic to nuclear as is consistent with the nuclear export inhibitor action of the drug. GFP and GFP-wtNPM do not show any visible change in subcellular localisation. Bar=30μM. Adapted from Chan and Lim 2015.

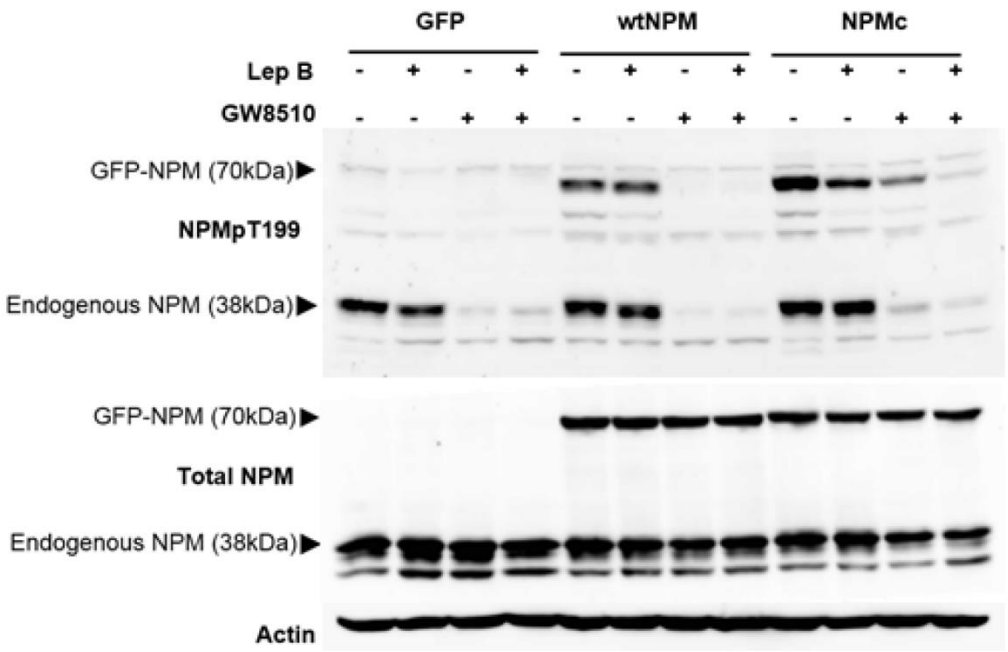


**Figure 4.5 Relocalisation of NPMc decreases T199 phosphorylation.**
Cells were treated with leptomycin B as per figure 2 and western blotted. Treatment with leptomycin B alone reduced T199 phosphorylation of NPMc but not wtNPM whereas cdk2 inhibitor, GW8510 (5μM, 24 hrs) affected both wtNPM and NPMc. Co-treatment of leptomycin B with GW8510 further reduced the phosphorylation of NPMcT199. Lep B, leptomycin B; GW8510, cdk2 inhibitor. GFP, HEK293T stably overexpressing GFP; wtNPM, HEK293T stably overexpressing GFP-wtNPM; NPMc, HEK293T stably overexpressing GFP-NPMc. Adapted from Chan and Lim 2015.

To investigate how the dephosphorylation of NPM might be facilitated, the localisation of protein phosphatase 1 β (PP1β) was visualised using immunofluorescence (Figure 4.6). The nuclear localisation of PP1β suggests that the reduction in NPMc phosphorylation upon lepB treatment could be due to the accessibility of the phosphatase and NPM substrate upon relocalization of NPMc back into the nucleus. In untreated cells, the cytoplasmic pool of NPMc has a greater capacity to retain T199 phosphorylation as the majority of PP1β is physically isolated from it in the nucleus.

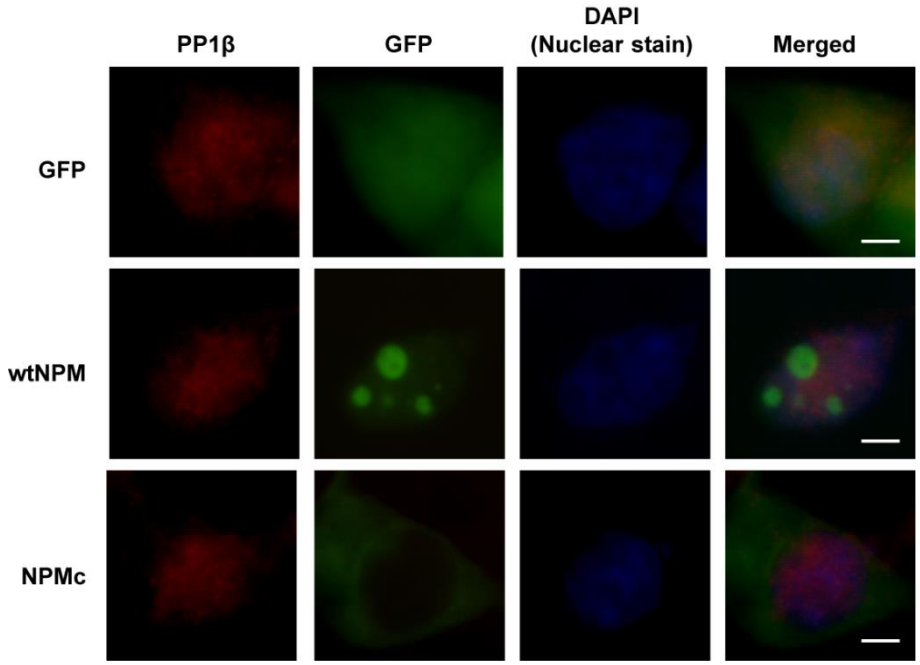


**Figure 4.6 PP1β is localised to the nucleus.**
Immunofluorescence staining of PP1β in HEK293T overexpressing GFP, wtNPM or NPMc. Cells were fixed in paraformaldehyde and stained with rabbit anti-PP1β followed by anti-rabbit Alexa fluor 568 conjugate. Bar=10μM.

### 4.2.3 More NPMc is phosphorylated earlier in the cell cycle than wtNPM

As the movement of NPMc back into the nucleus was in itself sufficient to reduce phosphorylation, we hypothesise that the increased phosphorylation of NPMc is caused by the shear amount of NPM in the cytoplasm that needs to be phosphorylated during centrosome licensing. Immunofluorescence staining of NPMphosphoT199 shows that there is widespread phosphorylation of NPMc in the cytoplasm of NPMc overexpressing cells prior to nuclear breakdown causing a doughnut shaped staining pattern, whereas phosphorylation is only visible after nuclear breakdown in wtNPM and GFP overexpressing cells (Figure 4.7). This is consistent with the hypothesis since prior to nuclear breakdown in wtNPM-expressing cells, there would only be a small amount of NPM in the cytoplasm inhibiting centrosome duplication so only a small amount of phosphorylation is needed which is not visible. However in the NPMc-expressing cells, much more NPM in the cytoplasm means that at the same stage in the

cell cycle when centrosome duplication is being initiated, more phosphorylation needs to occur. After nuclear breakdown, the NPM in the nucleus is no longer spatially restricted from preventing centrosome duplication so all the NPM is phosphorylated such that the phosphorylation is visible in both wtNPM and NPMc expressing cells.

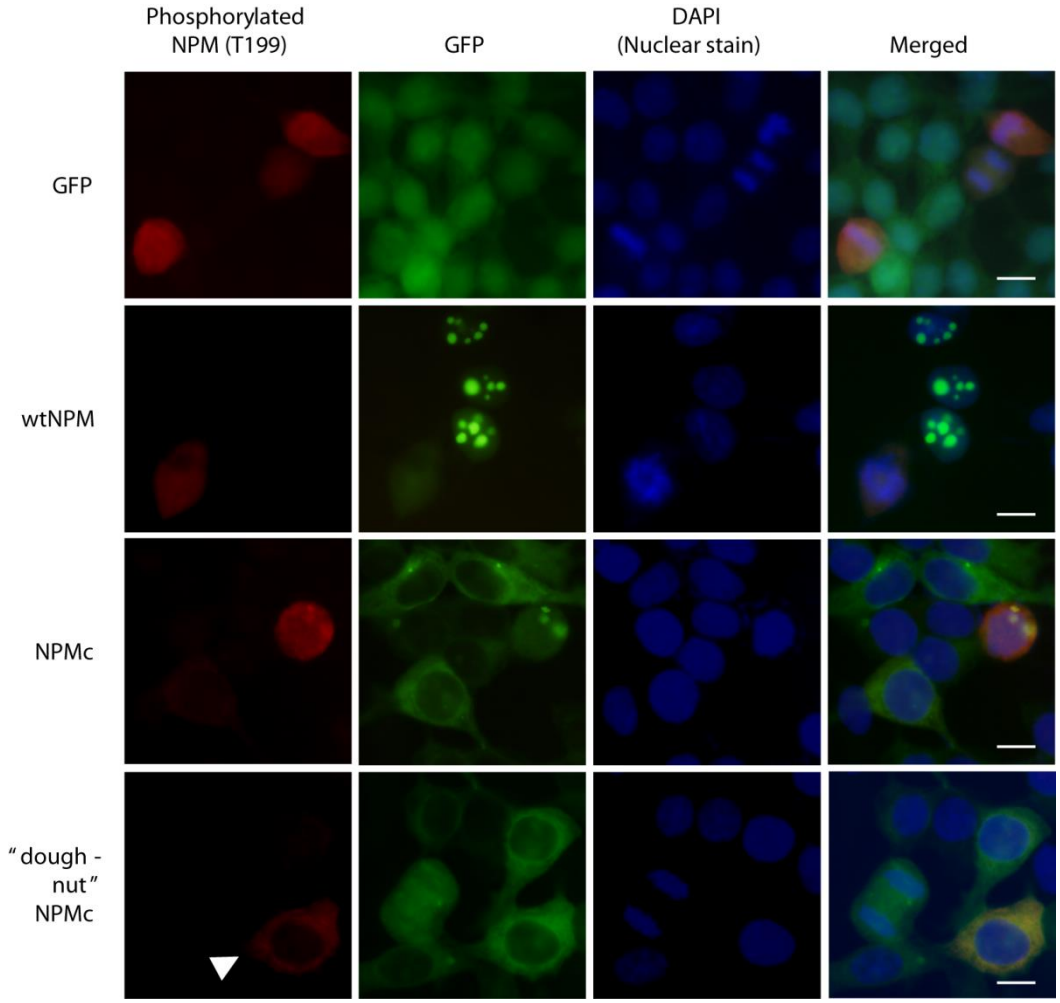


**Figure 4.7 NPMc is phosphorylated earlier in the cell cycle than wtNPM.**
HEK293T stably expressing GFP, GFP-wtNPM (wtNPM) or GFP-NPMc (NPMc) were stained with rabbit anti-phosphoNPM(T199) followed by anti-rabbit Alexa fluor 568 conjugate. Unlike wtNPM and GFP expressing cells, immunofluorescence staining shows that cells expressing NPMc have phosphorylation before nuclear membrane dissolution resulting in a "doughnut" shaped staining pattern around the nucleus (arrowhead). Bar=20μM. Adapted from Chan and Lim 2015.

## *4.3 Discussion*

While there is a wealth of information on wtNPM's centrosome function (Tokuyama et al. 2001; Tarapore et al. 2002; Okuda 2002; Cha et al. 2004; Wang et al. 2005; Shinmura

et al. 2005; Ma et al. 2006; Brady et al. 2009; Krause and Hoffmann 2010; Adon et al. 2010; Ferretti et al. 2010; Yao et al. 2010; Wang et al. 2011; Reboutier et al. 2012; Xia et al. 2013) and NPMc's role in AML (Alcalay et al. 2005; den Besten et al. 2005; Gurumurthy et al. 2008; Cilloni et al. 2008), there remains a gap in the literature of NPMc's effects on the centrosome especially in the light of its correlation with normal karyotype (Di Fiore 2008).

### 4.3.1 Possible causes of disparate phosphorylation

This chapter has eliminated kinase expression levels and localization as a possible cause of increased NPMc phosphorylation (Figure 4.1 and Figure 4.2). Activation of the kinase is also unlikely to be responsible. NPMc-overexpressing cells had a lower level of cyclin E than GFP or GFP-wtNPM (Figure 4.1). This is counterintuitive because one might expect cyclin E which is an activator of cdk2 to be increased in order to stimulate cdk2's kinase activity, thereby increasing NPMcT199 phosphorylation. The unvarying levels of other cyclins, cdks and phosphatase also fail to explain the increased phosphorylation of NPMc but it is the nuclear localisation of the phosphatase PP1β (Figure 4.6) that can explain the phosphorylation difference between nuclear wtNPM and cytoplasmic NPMc. This hypothesis would be supported if expression of a mutant PP1β with a nuclear export signal increased T199 phosphorylation of nuclear NPM. It would also be interesting to see whether other cdk2 targets are affected by the increased workload of NPMc phosphorylation. Since cdk2 is not upregulated in quantity or activity, the phosphorylation of other targets may be less efficient as they have more NPM to compete with.

NPMc's elevated phosphorylation could also be because of other activators of cdk2. RAB-like 6 isoform A (RABL6A) is known to interact directly with NPM and prevents

cdk2-mediated T199 phosphorylation. Knockdown of RABL6A induces centrosome amplification and increased NPMpT199 phosphorylation (Zhang et al. 2013). It would be interesting to see if and how this interaction is affected in NPMc.

Another aspect yet to be explored is that NPMc may have a different binding capability to cdk2 than wtNPM. Immunoprecipitation would be able to show whether the C-terminal mutation structurally affects NPM's upstream portion to the point of affecting binding affinity to cdk2. It could be that the structural change of NPMc facilitates its increased phosphorylation by having a greater propensity for phosphorylation.

While phosphorylation of both wtNPM and NPMc decreased in response to cdk2 knockdown or chemical inhibition, only NPMc responded significantly to leptomycin B treatment, emphasising that localisation is the most likely cause of NPMc hyperphosphorylation. However, several questions remain about whether T199 phosphorylation could confer increased protein stability as NPMc does not seem to be degraded in the cytoplasm unlike other proteins such as ARF and Fbw7γ. This issue should be addressed in future studies.

### 4.3.2 CRM inhibitors and NPM

Leptomycin B (lepB) works by inhibiting the nuclear export protein CRM1 (chromosome region maintenance; also known as XPO1, Exportin-1). Originally found to be produced by *Streptomyces sp.* (Hamamoto et al. 1983), lepB occupies ~70% of the groove in CRM1 where the nuclear export signal (NES) of the cargo protein would normally sit (Sun et al. 2013). Once proposed as an anti-tumour agent, lepB's cytotoxity lead to its discontinuation in clinical trials (Newlands et al. 1996) but it still remains a valuable research tool today due the relevancy of nuclear export inhibitors (NEI) in cancer treatment. A number of NEIs have shown to be promising in clinical trials for

both haematological and solid tumours and AML is no exception (Ranganathan et al. 2012; Gravina et al. 2014). Unlike the HEK293T overexpression system (Figure 4.4), treatment of AML3 with CRM1 inhibitor, KPT-185, results in nucleolar, not just nuclear, relocalisation. This is most probably because there is enough wtNPM in AML3 to form hetero-oligomers with NPMc and pull the NPMc from the nucleoplasm to the nucleolus. In the HEK293T overexpression system, the amount of NPMc greatly outweighs wtNPM so even when NPMc re-enters the nucleus upon CRM1 inhibition, there is not enough wtNPM to overcome the lack of NoLS in the exogenous NPMc. This is supported by previous experiments showing that wtNPM and NPMc exist in a 'dose-dependent tug of war' where localisation changes in concordance with titration of the two opposing NPM species (Bolli et al. 2009). Therefore, future experiments should focus on how the wtNPM:NPMc ratio affects NPM phosphorylation.

Another CRM1 inhibitor, a recently identified fungal derivative, avrainvillamide (Wulff et al. 2007), has been shown to not only bind and inhibit CRM1 but also NPM itself, restoring nucleoplasmic as well as nucleoli localisation. Initially isolated from a tropical marine fungus (Fenical et al. 2000), avrainvillamide forms a reversible S-alkylation bond with cysteine 275 (C275) of NPM. It binds 4.6 times faster to NPMc than wtNPM, probably due to the more unfolded state of the mutant making C275 more accessible. The additional cysteine 288 introduced by NPMc's (mutant A, Figure 1.5) contributes minimally to drug binding (Mukherjee et al. 2014). The fact that avrainvillamide can bind to NPM's C-terminus and induce nucleolic localisation implies that at least some of the tertiary structure of the nucleoli localisation signal is restored even though both tryptophans that form its hydrophobic core have been deleted. A 3D crystal or NMR structure of the drug and NPM would be invaluable in shedding light on how the disordered C-terminus is affected by avrainvillamide.

The cellular impact of such drugs is complicated by the dual role of CRM1 as a nuclear exporter as well as a centrosome localiser of which both functions are dependent on the same nuclear export signal (NES) of the cargo protein. Therefore CRM1 inhibitors like lepB would have a twofold impact on NPMc: 1) nuclear localisation and 2) inability for centrosome localisation. Avrainvillamide claims to do much more. Because this electrophilic antiproliferative drug binds to both NPM and CRM1, it not only perpetuates the two effects of CRM1 inhibition stated above but also restores C-terminal structure leading to nucleolar localisation and inhibits protein phosphatase 1β which increases T199 phosphorylation, further exasperating the lack of NPM at centrosomes.

### 4.3.3 Phosphorylation and centrosomes

In addition to the restoration of wild-type nucleoli localisation, Mukherjee et al. (Mukherjee et al. 2014) document the dissociation of NPM away from centrosomes leading to overduplication. This response is similar to a previous report on lepB in which the authors suggest that, in addition to nuclear export, CRM1 also enriches NPM at centrosomes preventing duplication. Mutation of threonine 95 (T95) to the phospho-mimetic aspartate within the NES of NPM prevents NPM's association with CRM1 and leads to centrosome overduplication (Wang et al. 2005). By contrast, Mukherjee et al. provide evidence that NPM's disassociation with centrosomes is the result of an increase in T199 phosphorylation caused by avrainvillamide's interaction with NPM, preventing it's dephosphorylation by PP1β and not by any direct interaction with PP1β itself. Since neither Mukherjee et al. nor Wang et al. report the phosphorylation states of T95 or T199 (or any other phosphorylation site for that matter) respectively, it is difficult to tell whether there is some kind of interaction between these two (or other)

phosphorylation sites and how they both contribute to centrosome regulation. T199 phosphorylation has only recently been shown to be dependent on another phosphorylation site, serine 4 (Ling et al. 2015) so it is clear that more than 10 years after the discovery of NPM's role in centrosome duplication, there are many complexities yet to be illuminated of which the next chapter of this thesis can only begin to explore.

# 5 Consequences of increased NPMc phosphorylation

## *5.1 Introduction*

The centrosome is a non-membrane bound organelle consisting of two orthogonally arranged centrioles surrounded by electron-dense pericentriolar material. As the main microtubule-organizing centre of animal cells, it is responsible for nucleating spindle poles for the separation of duplicated DNA in mitosis so duplication of DNA and centrosome are coupled in most cells, both being triggered by the cyclin-dependant kinase (cdk) 2/cyclin (cyc) E complex which is active in mid-late G1 phase of the cell cycle (Okuda 2002). Without coordinated centrosome amplification, cells may end up with mono- or multi-polar spindle assemblies, causing improper chromosome segregation and ploidy, thus contributing to genomic instability (Delattre and Gönczy 2004).

Genomic instability has been described as one of the hallmarks of cancer (Hanahan and Weinberg 2011) and acute myeloid leukaemia (AML) is no exception with 50-60% of cases having an abnormal karyotype. Of those with normal karyotype, 61.7% (Falini et al., 2005) heterozygously (Quentmeier et al. 2005) express a mutant form of nucleophosmin (NPMc; Falini et al., 2009) that is delocalized to the cytoplasm in contrast to the wild-type (wt) which is predominantly localized in the nucleolus (Di Fiore 2008). NPM has been implicated as an inhibitor of centrosome duplication. $NPM^{-/-}$ fibroblasts develop tetraploidy and aneuploidy caused by centrosome overamplification (Grisendi et al. 2005) and depletion of NPM in the cytoplasm using the nuclear export inhibitor leptomycin B also induces supernumerary centrosomes (Shinmura et al., 2005). Despite the fact that NPMc has greater accessibility to the centrosome as both localised in the cytoplasm, it still remains a largely untapped topic. The previous chapter demonstrated the link between NPM localisation and

phosphorylation. This chapter aims to further advance this link with respect to cellular proliferation, the means by which malignant cells increase their number, disrupting the homeostasis of the body.

## *5.2 Results*

### 5.2.1 Phosphorylation inhibition causes greater G2/M arrest of cells with only one centrosome in NPMc-overexpressing cells than wtNPM and GFP counterparts

A cell cycle analysis of the effects of cdk2 inhibition on synchronised cells (Figure 5.1) was consistent with previous results on non-synchronised cells (Figure 3.6, upper panel): The number of cells in G2/M and S phase increased with a corresponding decrease in G0/G1. However, the effect is augmented in synchronised cells as most cells are in a similar cell cycle stage and hence respond similarly unlike non-synchronised cells where the effect may be masked by different cells in different cell cycle stages.

Only in NPMc-overexpressing cells does the number of G2/M phase cells exceed those in G0/G1 upon GW8510 treatment (Figure 5.1) indicating that NPMc-overexpressing cells are much more dependent than GFP or wtNPM controls on cdk2 activity for mitotic progression.

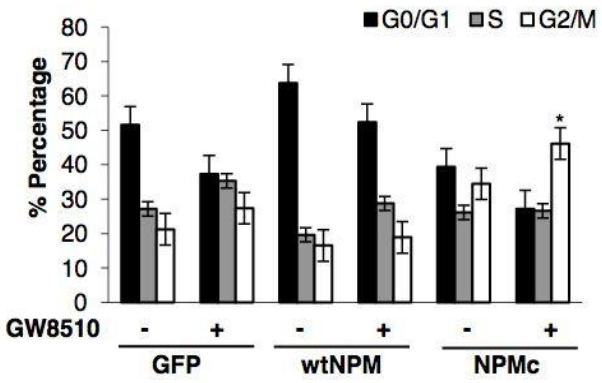


**Figure 5.1 Cdk2 inhibition causes G2/M phase arrest**
Cells were synchronized by serum starvation for 72 hours and then released into complete media containing GW8510 or the control solvent dimethyl sulfoxide (DMSO) for 24 hours.

Statistical significance was calculated using student's T-test in comparison to DMSO controls. * $p<0.05$. Adapted from Chan and Lim 2015.

To monitor the state of centrosomes during such a cell cycle arrest, cells were fixed and stained with the centrosome-specific marker protein γ-tubulin followed by a fluorescence-conjugated secondary antibody (Figure 5.3). The visualised centrosomes could then be tallied and the number of cells with one, two or more than two centrosomes counted.

Despite the obvious difference in cell cycle response to cdk2 inhibition, centrosome numbers did not change significantly for both synchronized and non-synchronized cells (Figure 5.3), indicating that the centrosome and DNA replication cycle were decoupled. The increase in 4n cells without a concurrent increase in cells with 2 centrosomes upon cdk2 inhibition suggests that the DNA has completed its duplication but not the centrosome thus preventing mitotic progression causing the accumulation of cells in G2/M phase. As NPMc-overexpressing cells have the greatest number of arrested cells, this suggests that cells with NPMc have a greater dependency than wtNPM on cdk2 activity and T199 phosphorylation for centrosome duplication and mitotic progression.

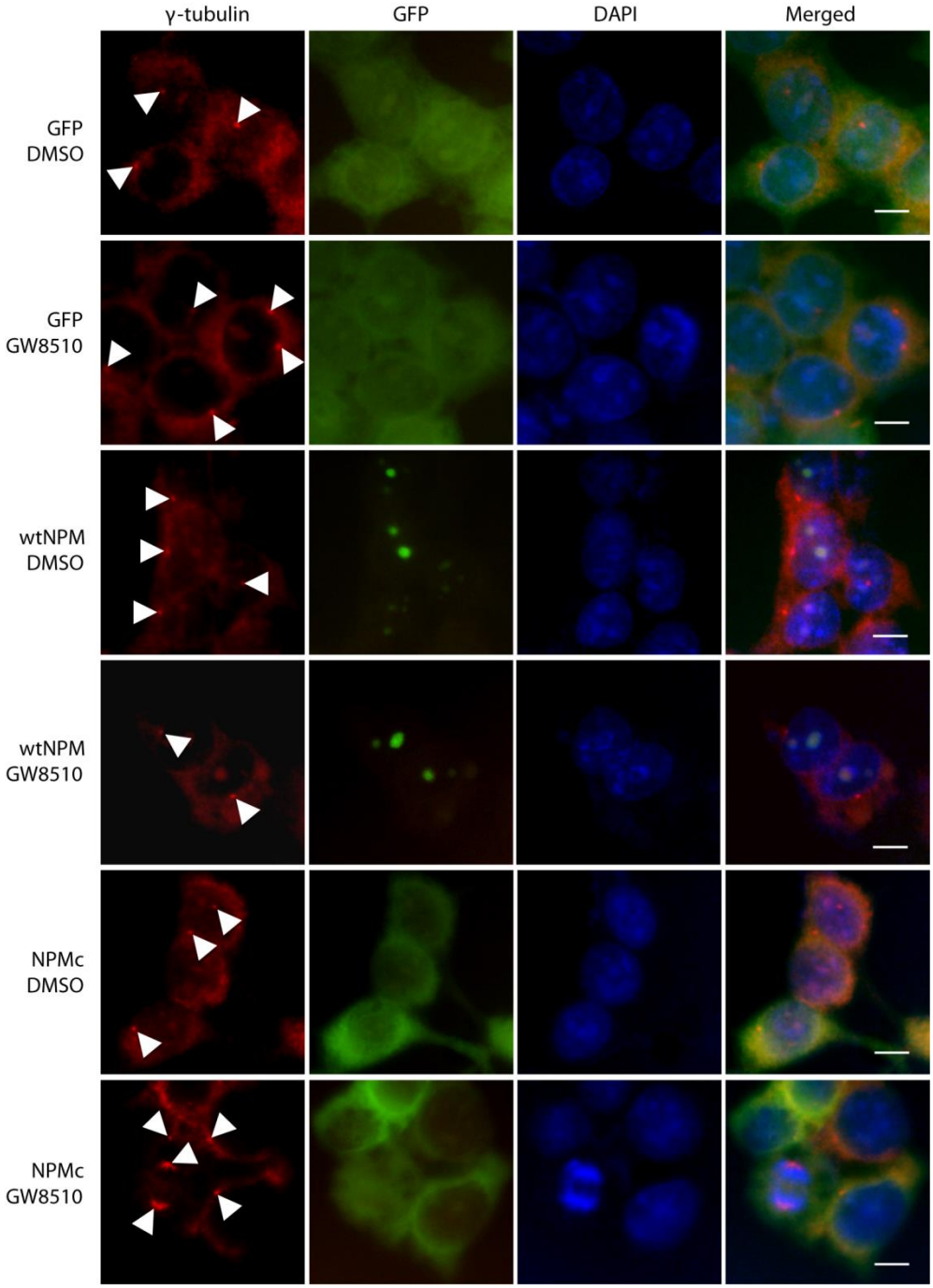


**Figure 5.2 Examples of centrosome staining in HEK293T stable cell lines under control (DMSO) or GW8510 treatments.**

Centrosomes appear as distinct red puncta (arrowheads) which can be counted. HEK293T stably expressing GFP, GFP-wtNPM (wtNPM) or GFP-NPMc (NPMc) were stained with rabbit anti-γ-tubulin followed by anti-rabbit Alexa fluor 568 conjugate. Bar=20μM. DMSO, cells treated 0.1% v/v of the solvent dimethyl sulfoxide as control for 24 hours; GW8510, cells treated with 5μM of the cdk2 inhibitor for 24 hours; GFP, HEK293T stably overexpressing GFP; wtNPM, HEK293T stably overexpressing GFP-wtNPM; NPMc, HEK293T stably overexpressing GFP-NPMc. Adapted from Chan and Lim 2015.

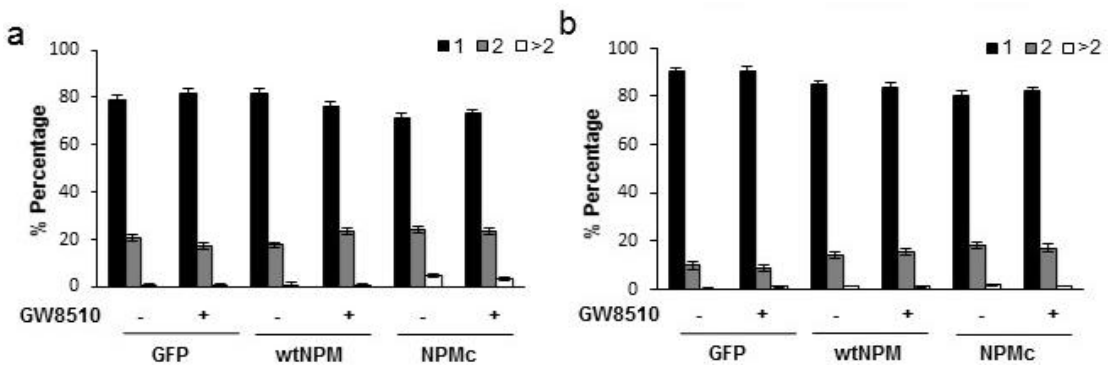


**Figure 5.3 Centrosome numbers do not change significantly upon cdk2 inhibition**
Centrosome numbers in non-synchronized cells (a) and synchronized cells (b). Cells were treated with or without 5μM of cdk2 inhibitor GW8510 for 24 hours and then fixed and stained for γ-tubulin protein which is specific to centrosomes. 1, cells with 1 centrosome; 2, cells with 2 centrosomes; >2, cells with 3 or more centrosomes. Results show mean and standard error from three independent experiments. Adapted from Chan and Lim 2015.

### 5.2.2 Serine 4 is also disparately phosphorylated

As further evidence that the disparate T199 phosphorylation of NPM is primarily due to its localisation with respect to the centrosome, the state of other NPM phosphorylation sites was investigated via western blot. Of the other centrosome related NPM phosphorylation sites, only serine 4 had a similar discrepancy as T199. Both phosphorylation of serine 125 which is associated with NPM homooligomerisation (Xia et al. 2013) and threonines 234/237 (T234/7) which are associated with the centrosome during mitosis (Cha et al. 2004) did not differ between wtNPM and NPMc (Figure 5.4). We propose that as the nuclear envelope breaks down during mitosis, this removes the relevancy of NPM's cellular localisation to the phosphorylation of the mitotic T234/7 site because all cellular NPM regardless of whether it is wild type or mutant is no longer physically separated from the centrosome resulting in similar phosphorylation levels. Serine 4 and T199, however, are phosphorylated pre-mitosis (Ling et al. 2015) when the nuclear envelope separates wtNPM and the centrosome. In this case, the different subcellular locales of wtNPM versus NPMc become relevant in determining the level of pre-mitotic phosphorylation due to their different accessibilities to the centrosome.

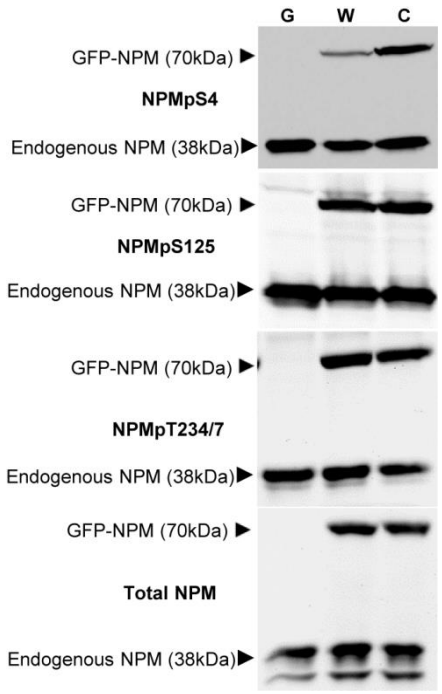


**Figure 5.4 Western blot of selected phosphorylation sites**
Pre-mitotic phosphorylation site serine 4 (NPMpS4) also shows similar disparity between wtNPM and NPMc but there is no such difference for other centrosome related sites serine 125 (NPMpS125) or threonines 234 and 237 (NPMpT234/7). G, HEK293T stably overexpressing GFP; W, HEK293T stably overexpressing GFP-wtNPM; C, HEK293T stably overexpressing GFP-NPMc.

### 5.2.3 Alanine-mutated NPMc mimics G2/M phase arrest caused by cdk2 inhibition

To show that G2/M phase arrest was not due to unspecific effects of the drug, site-directed mutagenesis was used to change T199 or S4 to non-phosphorylatable alanine. HEK293T cells were then transfected with either GFP-NPMc or the mutated GFP-NPMcT199A/S4A and treated with GW8510 before cell cycle analysis using flow cytometry. Western blot shows that the T199A mutant is undetectable by the phospho-T199 antibody (Figure 5.5b) and the localization of the T199A and S4A mutants were cytoplasmic showing that the mutation did not affect the localization of GFP-NPMc (Figure 5.5a). In addition, T199 phosphorylation of NPMcS4A and S4 phosphorylation of NPMcT199A mutant did not differ greatly from the non-mutated NPMc indicating that phosphorylation inhibition of either site did not affect phosphorylation of the other site. However, inhibition of NPMcT199 phosphorylation by site-directed mutagenesis

caused a cell cycle arrest similar to drug treatment indicating that the increase in G2/M phase cells is not due to any indirect effect of GW8510 (Figure 5.5c). Transfection of NPMcS4A also resulted in a G2/M phase arrest albeit more severe.

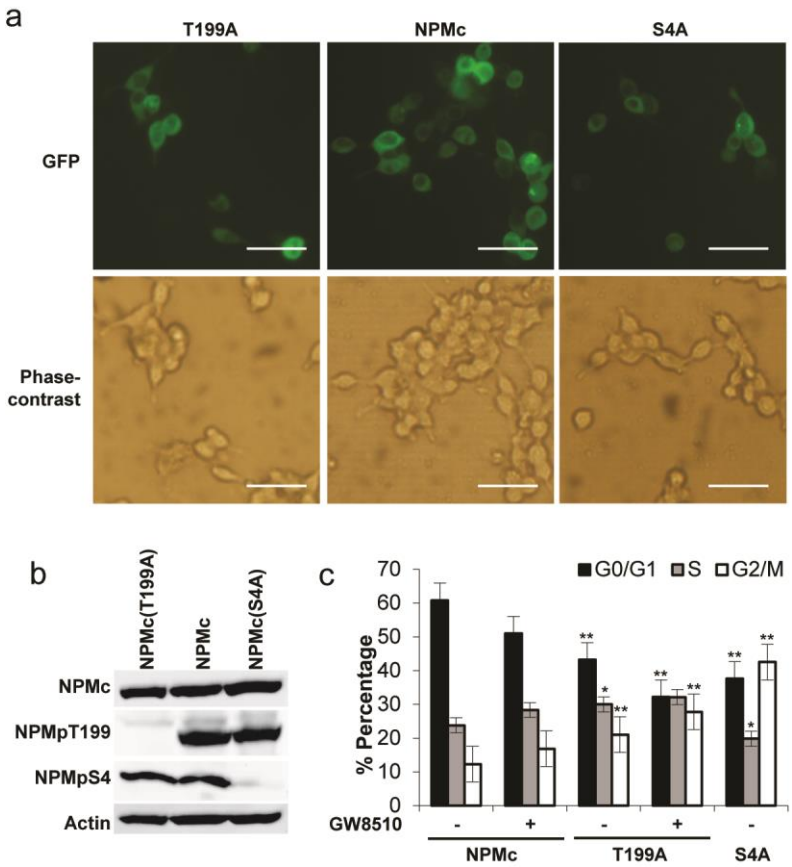


**Figure 5.5 Alanine-mutated NPMc mimics G2/M phase arrest caused by cdk2 inhibition.** (a) Light microscope images of HEK293T transfected with GFP-NPMc (NPMc), GFP-NPMc with mutated T199A (T199A) or GFP-NPMc with mutated S4A (S4A). All mutants have the same subcellular localization (cytoplasm) as NPMc. Bar=100µM. (b) Western blot shows that NPMcT199A and NPMcS4A are not detectable by antibody specific for phosphorylated NPM (NPMpT199 and NPMpS4, respectively). (c) HEK293T were transfected with GFP-NPMc (NPMc), GFP-NPMc with mutated T199A (T199A) or GFP-NPMc with mutated S4A (S4A) and treated with GW8510 or the solvent DMSO for 24 hours 24 hours post-transfection. Results show mean and standard error from three independent experiments. * p-value < 0.05, ** p-value < 0.01. Statistical significance was calculated using student's T-test compared to the respective NPMc treatment. G0/G1, cells with 2n DNA content; S, cells synthesizing DNA; G2/M, cells with 4n DNA content. Adapted from Chan and Lim 2015.

### 5.2.4 NPMc-expressing AML cell line is more sensitive to dephosphorylation inhibition

OCI-AML3 (AML3) is the only AML cell line that has so far been found to heterozygously express the NPMc mutation (Quentmeier et al. 2005). Immunofluorescence staining of NPM in AML3 shows that NPM is cytoplasmically localised in contrast with another AML cell line (OCI-AML2) which bears nuclear localized wtNPM24 (Figure 5.6a). Western blot of selected centrosome-related phosphorylation sites show that while T95, S125 and T234/7 were not disparately phosphorylated, the pre-mitotic phosphorylation site S4 had higher phosphorylation in AML3 compared to AML2 (Figure 5.6b). This further supports the importance of NPM's accessibility to the centrosome which is restricted by the nuclear envelope in pre-mitotic wtNPM-expressing cells. By contrast, we posit that in NPMc-expressing AML3, NPMc's ready access to the centrosome leads to compensatory hyperphosphorylation to prevent too much NPM binding to the centrosome which would inhibition centrosome duplication and cell proliferation.

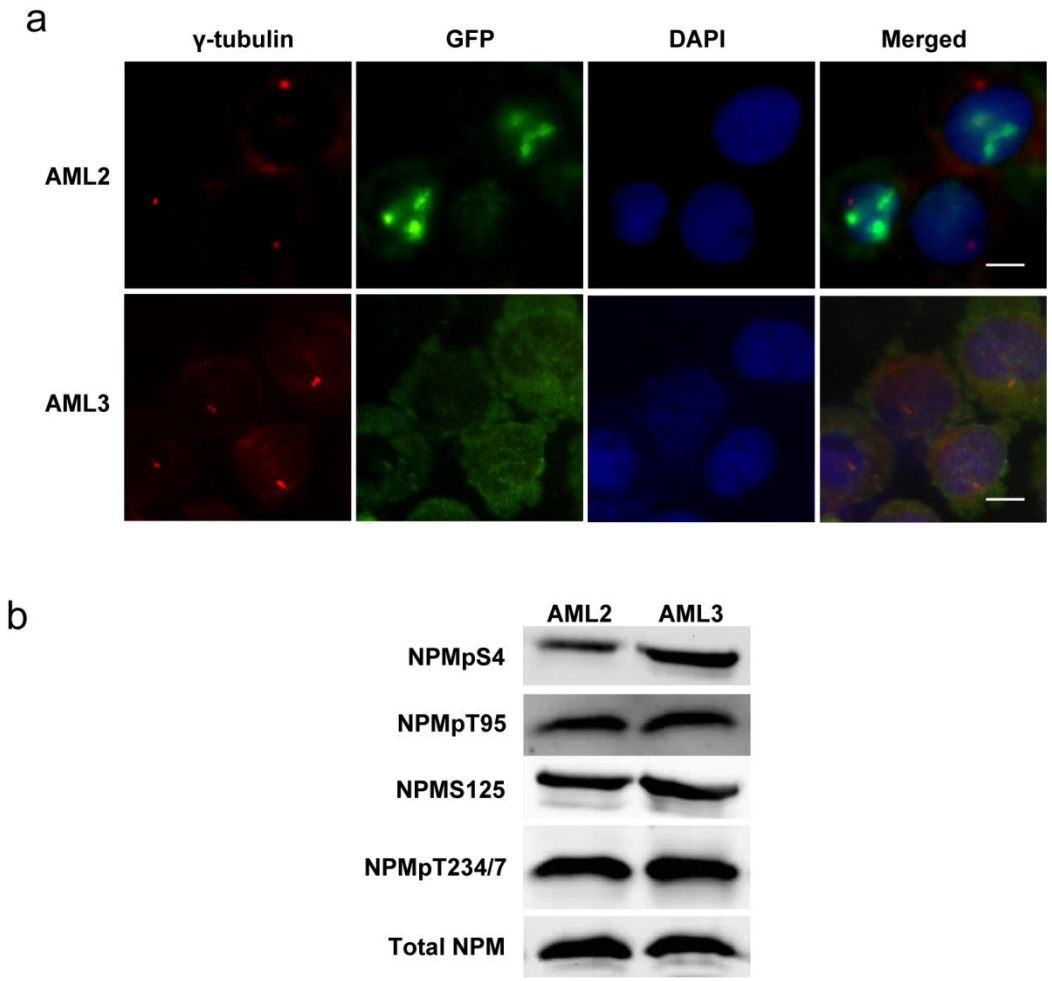


**Figure 5.6 S4 is likewise hyperphosphorylated in NPMc-expressing cell line OCI-AML3.** (a) Immunofluorescence co-staining of centrosome marker γ-tubulin (red puncta) and NPM (green) show that OCI-AML3 (AML3) has cytoplasmic NPM while OCI-AML2 (AML2) has wtNPM which is localised in the nucleus and separated from the centrosome by the nuclear envelope. Bar=10μM. (b) Western blot of selected NPM phosphorylation sites. S4 is more phosphorylated in AML3 compared to AML2. Other phosphorylation sites are not significantly different. Adapted from Chan and Lim 2015.

To see what impact dephosphorylation inhibition would have on NPMc- versus wtNPM-expressing cells, AML2/3 were treated with PP1 inhibitors tautomycin and calyculin A. Both cell lines responded similarly in a dose-dependent manner to drug treatment (Figure 5.7, upper panel): The phosphorylation of T199 increased. Furthermore, both drugs induced double strand breaks as evidenced by activated H2A.X (Figure 5.7, lower panel, orange line) which becomes phosphorylated on serine 139 (Podhorecka et al. 2010).

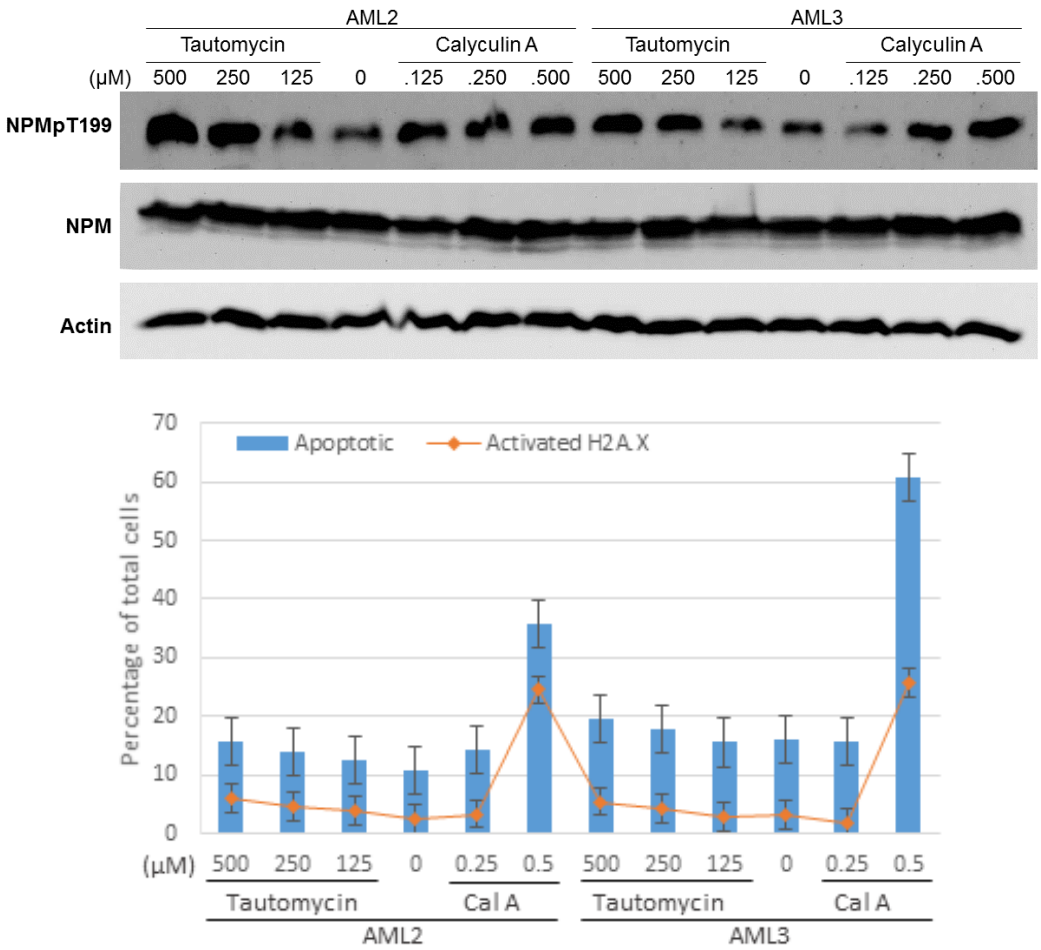


**Figure 5.7 NPM hyperphosphorylation is correlated with genome instability and cell death.**
Upper panel, Western blot of NPMpT199 in response to PP1 inhibition by the drugs tautomycin and calyculin A for 24 hours. Lower panel, correlation of cell death with DNA damage caused by PP1 inhibition. Activated H2A.X was assayed used immunostaining of phosphorylated serine 139 on H2A.X and quantifying the subsequent fluorescence using flow cytometry. Apoptotic cells were assayed using annexin V and PI staining. Any cell negative for both stains was considered live. Cells that were positive for either or both annexin V and PI stains were considered apoptotic. Data are representative three independent experiments, averaged. Error bars represent standard error.

Despite similar levels of H2A.X activation, AML3 had a higher number of apoptotic cells for both control and treated samples. This could be because AML2 and AML3 have different p53 statuses: the former overexpresses mdm4 which sequesters p53 in the cytoplasm as an inactive form (Tan et al. 2014). Therefore p53 is AML3 has greater capability than AML to be activated and trigger cell death.

## 5.3 Discussion

Since 2005 when the NPMc mutation was discovered, there has been a great dearth on its impact on centrosome duplication. Only recently has there been some foray into the relationship between NPMc phosphorylation and centrosome duplication (Mukherjee et al. 2014). Even though Mukherjee et al. show that T199 is affected in treated NPMc+ AML, the paper fails to compare the relative levels of phosphorylation in NPMc and wtNPM. Furthermore, there is no attempt to evaluate the impact of other phosphorylation sites. So while the prospect of nucleoli relocalisation is an exciting development, much more work still needs to be done to understand the resulting centrosome overduplication of avrainvillamide and leptomycin B (lepB) treatment (Wang et al. 2005; Mukherjee et al. 2014).

It is interesting that phosphorylation of the NES motif (T95) does not differ between AML2 and AML3 (Figure 5.6) since phosphorylation of this site abolishes CRM1 binding which would localise NPM to centrosomes. So T95 phosphorylation could be another strategy (in addition to S4 and T199) in which the NPMc+ cell avoids NPM inhibition of centrosomes. However, because CRM1 also exports NPM from the nucleus into the cytoplasm, T95 phosphorylation would also result in nuclear relocalization and the other oncogenic advantages of NPMc (Section 1.5.1) would be sacrificed. Therefore, to maintain proliferative advantage, other avenues of dissociating NPM away from the centrosome are employed. This is perhaps the reason why T95 phosphorylation is not disparate between AML3 and AML2.

Serine 125 is also not disparately phosphorylated. Phosphorylated S125 promotes oligomerisation of NPM which supports centrosomal localisation. Mutation of S125 to alanine induces centrosome amplification and phosphorylated S125 is only detected at centrosomes during G1 when there is one centrosome and not during S or G2 when

there are duplicated centrosomes (Xia et al. 2013; Shandilya et al. 2014). Therefore, reduced S125 phosphorylation would keep excess NPM away from centrosomes. However, this would also reduce oligomerisation and affect NPMc's ability to pull wtNPM out of the nucleus. Again, the phosphorylation of S125, like T95, affects not only centrosome regulation but also localisation. Hence, S125 is also not found to be instrumental in overcoming NPMc's centrosome duplication inhibition.

While the localisation of NPMcS125 and NPMcT95 phosphorylation mutants are yet unconfirmed, Figure 5.5 shows that mutation of S4 and T199 do not affect the localisation of NPMc. They are therefore more suited to be used to reduce NPM's inhibition of centrosome duplication, allowing mitosis to progress.

Furthermore, previous studies have indicated that wtNPM T199 phosphorylation is dependent on S4 phosphorylation as the S4A mutants have greatly reduced T199 phosphorylation (Ling et al. 2015). However, the authors did not evaluate the impact of a T199A mutant on S4. Figure 5.5b shows that for NPMc, mutation of T199 site has little impact on S4 and vice versa. Perhaps the cytoplasmic load of NPMc overrides the dependency relationship between S4 and T199. Clearly, more work is needed to elucidate the interplay of NPM's phosphorylation sites.

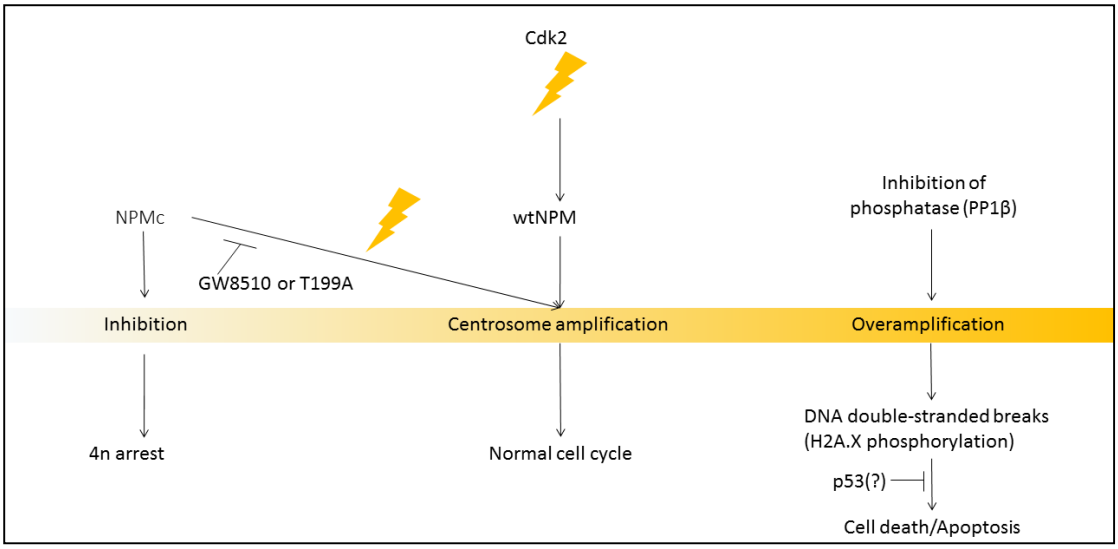


**Figure 5.8 Proposed Model.**
Intensity of yellow increases from left to right to symbolise increasing phosphorylation. Most of wtNPM is localised in the nucleus away from the centrosome so only an intermediate amount of phosphorylation by Cdk2 is needed to permit centrosome duplication. Too much unphosphorylated NPM in the cytoplasm, caused by drug treatment (GW8510) or site-directed mutagenesis (T199A), inhibits centrosome duplication and causes cell cycle arrest of cells with duplicated DNA (4n arrest). Therefore, extra phosphorylation is needed (yellow lightning). Inhibition of the phosphatase (PP1β) increases apoptosis and H2A.X phosphorylation, an indicator of DNA double stranded breaks which usually causes cell death by triggering p53.

### 5.3.1 NPM and other centrosome regulators

The evidence in this chapter alludes to the necessity of elevated phosphorylation as a compensatory mechanism for NPMc+ cells to permit centrosome duplication. Inhibition of this phosphorylation leads to cell cycle arrest (Figure 5.8). However, many questions about NPM(c)'s exact role in centrosome duplication remain unanswered. For example, Rho-associated kinase (ROCK2) is a downstream effector of NPM and its kinase activity is upregulated 5-10 fold following interaction with NPMpT199 (Ma et al. 2006; Ling et al. 2015). BRCA2 also interacts with NPM. Abrogation of this interaction leads to overamplification of centrosomes leading to multipolar mitosis and multinucleated cells (Wang et al. 2011). While the domains of BRCA2 and ROCK2 that interact with NPM are known (amino acids 639–1,000 and 6-553, respectively), the same cannot be said for NPM. Mapping NPM's interaction region with BRCA2/ROCK2 would help deduce the stoichiometry of this interaction and aid in

deciphering how increase in cytoplasmic NPM could affect these interactions and whether T199 phosphorylation directly affects binding or, instead, induces conformational change that changes propensity for BRCA2/ROCK2 interaction. BRCA2 itself is responsible for 76% of male and female familial breast cancer (Ford et al. 1998) which is commonly found to possess centrosomal abnormalities and genomic instability (Guo et al. 2007). Furthermore, there is evidence that benign tumours differ significantly in terms of centrosome (dys)regulation from malignant tumours (Kronenwett et al. 2005). Therefore, further work would be needed to elucidate exactly how NPMc changes the dynamic between ROCK2, BRCA2 and other centrosomal regulators.

BRCA1 is also an interaction partner of NPM and its mutations account for 80% of familial breast and ovarian cancers (Couch 2004). BRCA1-dependant ubiquitination of γ-tubulin inhibits centrosome duplication (Parvin 2009) and it would be interesting to see if NPMc's ubiquitination status is affected in light of its cytoplasmic localisation.

Therefore, further studies into NPMc's centrosomal role benefit not only AML in which the mutation is found but the results of such studies could also be valuable for other cancers with a predisposition to genomic instability.

Already, the targeting of NPM in animal models of multiple myeloma (MM) have been shown to induce cell death by increasing multipolar spindle in mitosis. NPM was found to be the target of a phthalimide derivative, 2-(2,6-diisopropylphenyl)-5-amino-1H-isoindole-1,3- dione (TC11). MM is inherently gnomically instable and has multiple centrosomes but works around this by clustering extra centrosomes into two spindle poles during mitosis creating pseudo-normal chromosome separation. NPM knockdown or inhibition with TC11 prevents centrosome clustering leading to p53-independent apoptosis (Shiheido et al. 2012; Matsushita et al. 2015).

T199 has also recently been implicated in hepatocellular carcinoma caused by hepatitis B virus (HBV). The HBV protein HBx is correlated with increased T199 phosphorylation which prevents caspase 3 cleavage of NPM enabling it to execute anti-apoptotic functions. HBx also interacts directly with NPM in the nucleolus, enhancing NPM acetylation and promoting ribosome production (Ahuja et al. 2015). Whether NPMc's other post-translational modifications are affected is also another interesting area of study that impacts the body of knowledge in other cancers.

# 6 Conclusion

The title of this chapter is somewhat of a misnomer because this thesis is only the beginning of exploring NPMc's relationship with the centrosome.

In 2000, Hanahan and Weinberg published a generalistic review of cancer in the journal Cell that has been cited more than 4000 times entitled "The Hallmarks of Cancer" stating six acquired capabilities of malignancy and proposed that these six capabilities are common to "most and perhaps all types of human tumors" (Hanahan and Weinberg 2000). However, in soluble tumours such as AML, the importance of two of these capabilities is less relevant compared to solid tumours: Sustained angiogenesis and Tissue invasion & metastasis. The remaining four capabilities (self-sufficiency in growth signals, insensitivity to antigrowth signals, limitless replicative potential and evading apoptosis, Figure 6.1) form the foundation of an imbalance between cell division and cell death that characterises the rate of proliferation found in cancer which disrupts the homeostasis of the 100 trillion or so cells in the human body (Alberts et al. 2002). As described in section 1.5, NPMc has been shown to both increase cell proliferation and inhibit cell death. This thesis shows that while NPMc can inhibit extrinsic cell death (Leong et al. 2010), it's impact on intrinsic cell death is limited as XIAP expression is not increased and caspase 3 and 9 activities are not decreased.

In 2011, Hanahan and Weinberg published a follow-up article entitled, "Hallmarks of cancer: the next generation" (Hanahan and Weinberg 2011). In addition to reviewing their previously stated six capabilities more comprehensively, the authors further expound four concepts into their model (Figure 6.2). Of these four concepts, it is interesting to note that only one is previously mentioned in the 2000 article: genome instability.

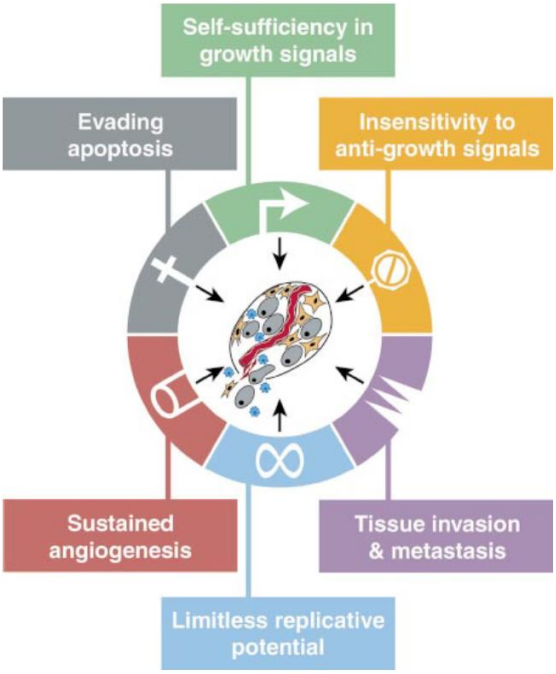


**Figure 6.1 The six hallmarks of cancer**
Adapted from Hanahan and Weinberg 2000.

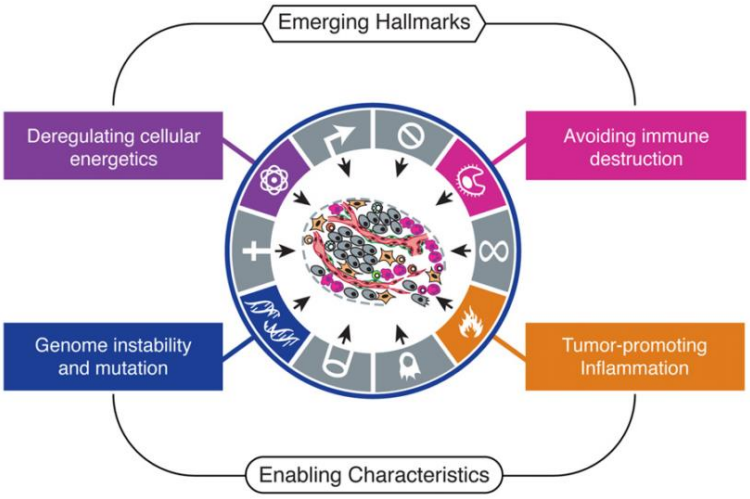


**Figure 6.2 Emerging Hallmarks and Enabling Characteristics**
The six capabilities mentioned in the 2000 article are in grey. Additional concepts introduced in the 2011 are in colour. Adapted from Hanahan and Weinberg 2011.

Because cancer requires multiple mutations to be manifested, genome instability is the means by which these hallmarks of cancer are acquired and this is attested to by the correlation between genome instability and poor prognosis (Chan 2011). However, genome instability is a double-edged sword: on the one hand, the genetic diversity

provided by genome instability is the basis for a Darwinian-like selection that encourages the survival of more aggressive cancer cells; on the other hand, too much genome instability increases the probability of deleterious mutations that trigger cell death and put a stop to cancer progression (Figure 6.3, Cahill et al 1999).

Cancer cells are thus like the female aphid in winter when conditions are harsher, resources are scarce and there is more selection pressure, they switch from asexual reproduction to sexual reproduction (Audesirk et al. 2002). Under such circumstances, the genetic diversity provided by sexual reproduction becomes advantageous in adapting to the changing environment. In summer, aphids take advantage of the abundance of food and do away with the hassles of seeking a mate and switch to more efficient asexual reproduction, parthenogenetically producing clones of themselves, akin to the process of mitosis on a cellular level. For the cancer cell, the source of genetic diversity is not sexual reproduction, as in the aphid, but genome instability. On this background of increased mutation, the cancer progenitor is able to overcome selection barriers such as cell death signals, antigrowth signals and differentiation signals.

The consequences of genome instability are as varied as the cancers they cause but they can be roughly divide into two categories: smaller scale DNA lesions such as point mutations, deletions and insertions which cannot be detected by fluorescence in-situ hybridisation; and larger scale karyotype changes such as chromosomal translocations and ploidy which can be caused by faulty double strand break repair and aberrant centrosome duplication (Nigg 2002). Again, as in the case of cell death, NPMc is selective in the way that it contributes to the genome instability arm of carcinogenesis. With 85% of NPMc+ AML being karyotypically normal, this points to a reliance on molecular mutations rather than chromosome aberrations as the source of malignant

transformation. NPMc is already known to negatively affect two methods of DNA repair which would prevent molecular mutations (Fantini et al. 2010; Ziv et al. 2014). However, it remains to be determined whether NPMc actually actively contributes to molecular mutations, in addition to preventing chromosome aberrations, in a manner that potentiates oncogenesis. Considering that NPM interacts with homologous recombination proteins BRCA1/2, it would be interesting to see if this repair pathway is also affected by NPMc.

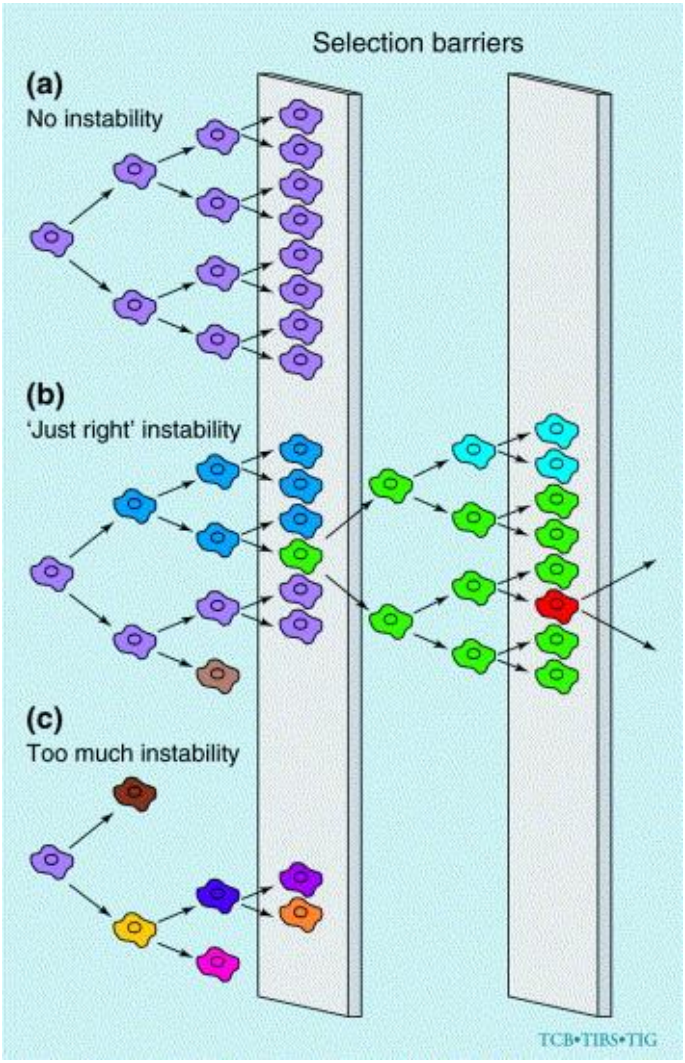


**Figure 6.3 The "goldilocks" trait of genome instability.**
Reprinted from Trends in cell biology, Vol 9, Daniel P Cahill, Kenneth W Kinzler, Bert Vogelstein, Christoph Lengauer, Genetic instability and darwinian selection in tumours, M57-M60, Copyright (1999), with permission from Elsevier.

## *6.1 Limitations*

While the results in this thesis hint at the impact of NPMc on the centrosome, there is not enough data to confirm its relationship to genomic instability. In order to ascertain this, more centrosomal experiments will need to be done in $p53^{-/-}$ cells. This is because in most cells with active p53, centrosome aberrations cause cell death making the study of centrosome effectors difficult. This thesis only assays change in centrosome numbers once (Figure 5.3) in overexpressing cell line HEK293T as they are easier to grow, maintain and manipulate than hematopoietic cell lines AML2/3. For example, immunofluorescence staining of AML2/3 requires that they are spun on glass slides since they do not adhere to coverslips. The mere centrifugal force of this process causes the cell to be somewhat squashed resulting in irregular cell membrane (AML3, GFP panel, Figure 5.6) and non-distinct nucleoli (AML2, GFP panel, Figure 5.6). Therefore more time and patience is required to optimise such a procedure to reduce cell deformities while still maintain enough centrifugal force to stick the cells to the glass slide.

Furthermore, analysis of cyclins (Figure 3.5, Figure 4.2 and Figure 4.3) was done in non-synchronised cell and since the levels of cyclins vary according to cell cycle stage, it may be necessary to assess whether differential expression of particular cyclins facilitate NPMc hyperphosphorylation at G1/S phase as alteration of cyclin expression in cultured/tumour cells is not unheard of (Erlandsson et al. 2003; Min et al. 2012).

In addition, dephosphorylation inhibition was done only using drugs which are non-specific (Figure 5.7). Tautomycin and calyculin A are both inhibitors of PP1 ($IC_{50}$~1nM) but also act against PP2A (Resjö et al. 1999). On top of this, PP1β is primarily responsible for NPMpT199 dephosphorylation and not α or γ isoforms but the drugs are unable to specifically target PP1β. Ideally, knockdown of PP1β would be

the most selective to evaluate the effects of dephosphorylation on centrosome numbers. Alternatively, T199 may be mutated to phospho-mimetics aspartate or glutamate as a complement to the alanine mutants in Figure 5.5.

The location of NPM(c) at centrosomes was also not examined thoroughly. Higher resolution confocal images would be needed to visualise the movement of NPM(c) and phosphorylation mutants at centrosomes in response to lepB treatment.

Lastly, centrosome abnormalities in animal models of NPMc which already exist (Bolli et al. 2010; Cheng et al. 2010) should be evaluated to review the extent of in vivo applicability.

## *6.2 Future work*

### 6.2.1 PIP3 signalling

It seems puzzling that in the 10 years since the discovery of NPMc, its centrosomal impact has been neglected until recently. Yet there is another seemingly obvious area of NPMc that has yet to be explored: Phosphatidylinositol 3,4,5-triphosphate. Phosphatidylinositol 3,4,5-triphosphate or PIP3 is well known secondary messenger in the cytoplasm known to activate pro-survival signalling pathways which are often mutated in cancer (Carnero et al. 2008). Wild-type NPM is already known to bind PIP3 in the nucleus and it's N-terminal 107 amino acids are dispensable for this binding (Ahn et al. 2005). Interestingly, NPM's C-terminal 56 amino acids also bind Akt's plecstrin homology (PH) domain in the nucleoplasm (Lee et al. 2008). Akt is a downstream effctor of PIP3 and its interaction with NPM stabilises the latter from caspase 3 cleavage, preserving its anti-apoptotic role. Nuclear PIP3 enhances NPM-Akt interaction but preferentially binds NPM and displaces Akt at higher concentrations (Kwon et al. 2010). NPMc could perhaps block the access of PIP3 and prevent the

activation of Akt. This would explain the a high level of ERK activation in NPMc-expressing cell line OCI-AML3 (Bte Ahmad 2014) as Akt is known to inhibit ERK activation by phosphorylating ERK's upstream kinase Raf (Zimmermann 1999; Guan et al. 2000; Moelling et al. 2002; Mendoza et al. 2011). Whether or not NPMc's mutation affects PIP3/Akt binding and dynamics in the cytoplasm remains to be ascertained and is an untapped area of study which would yield invaluable information about PIP3 signalling in AML.

### 6.2.2 Clinical prospects

Whether T199 or other phosphorylation sites of NPM can or should be targeted remains questionable. Since genomic instability is a driver for mutations, the cancer could theoretically develop new ways to advance more aggressively. On the other hand, most of such mutations are deleterious especially in cells with functional p53. AML3 has been shown to not deviate from canonical p53 (Tan et al. 2014) and most AML cases also have wild-type p53 (Hu et al. 1992).

Drugs targeting various aspects of centrosome duplication are already in clinical trials (Korzeniewski et al. 2012). It has been shown that cdk inhibitors prevent abnormal centrosome duplication but normal cell cycle is not disrupted (Tetsu and McCormick 2003; Duensing et al. 2004), therefore centrosome duplication inhibitors maybe more useful in cancer prevention rather than treatment per se. Even if NPMc's centrosome function is not feasible as a target in cancer treatment, it could still be used as a tool to study centrosome regulation.

## *6.3 Closing remarks*

While there are many barriers the cancer progenitor must overcome to achieve malignancy, the results in this study suggest that for NPMc+AML, the inhibition of

centrosome duplication is surpassed by hyperphosphorylation. In the process of eliminating XIAP as one of the oncogenic effects of NPMc, the centrosome related phosphorylation site T199 was found to be elevated compared to wild-type. This hyperphosphorylation was confined to pre-mitotic phosphorylation sites which is important as alteration of other phosphorylation sites are related to other cellular functions and may negatively affect NPMc's oncogenic advantages. Furthermore, downregulation of T199/S4 phosphorylation induced cell cycle arrest and inhibition of dephosphorylation increased double-stranded DNA breaks, a consequence of centrosome overamplification. As wtNPM's centrosomal role has enjoyed many years of attention, it's time that NPMc caught up with it.

# 7 References


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